PRACTICAL BACTERIOLOGY

PRACTICAL BACTERIOLOGY

AN INTRODUCTION TO
BACTERIOLOGICAL TECHNIC

BY
FRED W. TANNER, PH.D.
Professor of Bacteriology and Head of the Department
University of Illinois

SECOND EDITION

NEW YORK
JOHN WILEY & SONS, INC.
LONDON: CHAPMAN & HALL, LIMITED

SECOND EDITION
Sixth Printing, February, 1947

PRINTED IN U. S. A.

PREFACE

This laboratory guide for students who are beginning the study of bacteriology has been prepared to accompany the author's "Bacteriology." It may, however, be used with other texts. Direct references to text-books have not been given since it is assumed that a student who has reached the stage in his development where he is studying bacteriology should know how to use such books. Furthermore, no one author has a monopoly on the best methods for presenting information, and a student should be encouraged to consult as many of the sources of information as possible.

This laboratory guide is somewhat different from a number of recent ones which have been published. It may be no better. The author has avoided the presentation of the various procedures in the study of bacteria in exercise form. They will be found distributed through the book but easily accessible to students. In Chapter VII is given one arrangement by which the book may be adapted to a course meeting three times a week on alternate days. Other outlines may be very easily prepared for classes having other schedules. It has been the author's experience that practically no printed manual exactly fits the requirements of all laboratories. It is hoped, however, that this book may be easily adapted to various conditions.

No attempt has been made to include a mass of extraneous experimental work which should be, and usually is, covered in more advanced courses. The author, after twelve years' contact with students who are beginning their study of bacteriology, believes that an introductory laboratory guide should contain work which will make students proficient in ordinary technic.

An attempt has been made to avoid the use of pathogenic bacteria. There are very few places in an introductory course in bacteriology where a non-pathogenic microorganism cannot be found which will be just as useful as a pathogenic species. Some may believe that this will detract from the interest of the student

since he might be stimulated by the use of pathogens. Such an argument might justify the use of dynamite or trinitrotoluol in beginning courses in chemistry. Furthermore, the continued study of pathogens tends to give a new student a warped idea of the science. There are plenty of organisms and material with which to study the subject of bacteriology as a pure science and thus lay a firm foundation for the various structures of applied bacteriology which may be built later upon such a foundation.

F. W. TANNER

May, 1928

CONTENTS

CHAPTER I

GENERAL BACTERIOLOGICAL APPARATUS AND PROCEDURES

CHAPTER II

THE MICROSCOPE AND ITS USE

CHAPTER III

CULTURE MEDIA

CHAPTER IV

MICROSCOPIC EXAMINATION OF BACTERIA

CHAPTER V

ISOLATION OF BACTERIA

CHAPTER VI

PROCEDURES FOR STUDYING THE CHARACTERISTICS OF BACTERIA

CHAPTER VII

STUDY OF YEASTS

CHAPTER VIII

STUDY OF MOLDS

CHAPTER IX

PLANS FOR WORKING

CHAPTER X

SPECIAL EXERCISES

APPENDIX

The more I study nature, the more I stand amazed at the work of the creator. I pray while I am engaged in my work in the laboratory.

—PASTEUR.

PRACTICAL BACTERIOLOGY

CHAPTER I

GENERAL BACTERIOLOGICAL APPARATUS

THE apparatus used in a bacteriological laboratory may not necessarily differ from that used in a chemical or biological laboratory. The rigorous technic required to secure and maintain sterility of such apparatus and contents has made it necessary to introduce new procedures into the bacteriological laboratory, which are not used by students of other living organisms. Consequently, in certain cases, special devices have had to be introduced by the microbiologist. It is not the author's intention to discuss completely all the different types of apparatus which may be used. He believes that even those students who are beginning the study of bacteriology should know the more common pieces of apparatus with which they, as bacteriologists, will have to work. One of the best times to begin to become familiar with apparatus is at the beginning of instruction.

Quality of Glass.—This is a factor in the selection of apparatus to which more and more attention is being given. It is probably not within the scope of a book on introductory practical bacteriology to present a detailed review of the data which have been reported in this connection; however, reference may be briefly made to the fact that a number of grades of glass are being used in apparatus. The improved methods of Clark and Lubs for determining the reaction of culture media and similar materials have made it easier to follow changes in reaction due to substances dissolved from the glass. Esty and Cathcart showed that the heating of unbuffered solutions in soft-glass tubes greatly affected the hydrogen-ion concentration. The data presented in their paper leave little doubt that this question has

received too little attention. In general, hard-glass tubes were more satisfactory than soft-glass tubes.

Fabian and Stull also reported data from a similar investigation. "Glassware taken from stock, filled with a non-buffered solution as conductivity water, and autoclaved for thirty minutes at 15 pounds pressure, yielded enough alkali to change the reaction of the conductivity water from *p*H 7.0 to *p*H 9.8. The amount of alkali yielded by this same glassware was not sufficient to change the reaction of a buffered solution as nutrient broth. In fact, the nutrient broth with a reaction of *p*H 7.0 before autoclaving had a reaction in some cases of *p*H 6.8 after autoclaving in this glassware. Soft glass yielded more alkali upon autoclaving than did hard glassware." The data published in these two papers should convince one that for special bacteriological work, at least, the quality of glass may be important. For very careful work, apparatus made of resistant glass should be used.

The following general groups of glass may be recognized:

Hard Glass.—This is a resistant type of glass. Before the World War practically all of the world's supply of hard glass came from Jena, Germany, and consequently Jena glass was regarded as the perfection of quality in glassware. When the War cut off the supply of this glass, researches were carried out in America to find a substitute. We now have resistant glasses, such as Pyrex, made by the Corning Glass Works of Corning, N. Y., which are probably superior to any other glass made, either in America or in Europe, if stability, heat shock and mechanical shock are to be taken as indices of quality. Pyrex is a low-expansion borosilicate glass of simple chemical composition containing no metals of the magnesium-lime-zinc group and no heavy metals. Apparatus from this glass is becoming more common in the bacteriological laboratory.

Soft Glass.—In this group fall a number of types of glass which lack the qualities given above for hard glass. Apparatus made from this glass may cause changes in the hydrogen-ion concentration of the medium by allowing the solution of certain ingredients in the glass. Such glass, if used for the manufacture of apparatus which will be repeatedly sterilized, may etch and make observations on cultures more difficult. The fact that the glass in such apparatus is slowly dissolved is borne out by the white or etched appearance which is often seen below the meniscus line. However, culture tubes of soda-lime glass, which will stand repeated sterilization without appreciable decomposition, are available.

Cleaning Glassware.—The successful bacteriologist needs absolutely clean apparatus. There is no science in which success depends more on clean apparatus than in bacteriology. No special procedure needs to be outlined for cleaning apparatus.

All new apparatus should be thoroughly washed and should never be used in bacteriological work until this has been done. Alkali or acid materials may adhere to it and cause errors in observations since some salts tend to stimulate bacterial growth while others inhibit it. After thorough cleaning in water and soap, if necessary, the apparatus should be subjected to the action of a cleaning mixture if any material fails to be removed by the usual preliminary methods. This cleaning mixture may be prepared by dissolving 80 grams of potassium dichromate ($K_2Cr_2O_7$) in 300 c.c. of water, distilled if available; add this when cool to 460 c.c. of concentrated commercial sulfuric acid with constant stirring. This mixture may be stored in a lead-lined box where it will be easily and quickly available for use, or in glass-stoppered bottles. It should not be thrown away but returned to the original container after use, as long as it retains its first color. This cleaning mixture will remove any film of fatty substances and other organic matter.

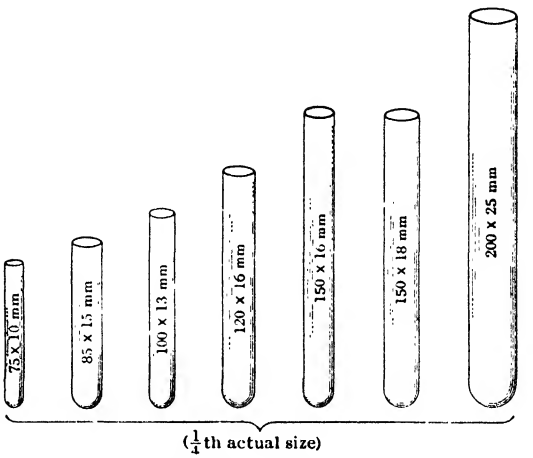


FIG. 1.—Showing Different Sizes of Culture Tubes without Lips for Bacteriological Work.

Culture Tubes.—The culture tubes which are used in bacteriology are much like the test tubes used in chemistry and other sciences. Those without the lips are preferred by some workers because they are easier to handle and pack better into baskets. This type is often called the " bacteriological " or " board of health " type of simple culture tubes. No special size culture tube is solely adapted to bacteriological work because the various sizes have their uses. The test tube that is used as the outer tube for the Durham fermentation tube should be sufficiently larger than the inner tube to allow the entrance of a needle or loop into the medium.

Petri Dishes.—The introduction of liquefiable solid media by Robert Koch made it necessary to have a dish into which the medium could be poured for hardening and protection from other microorganisms. Koch poured his gelatin on to sterile glass plates which were placed on a level surface to harden. These plates possessed no covers and consequently it was difficult to

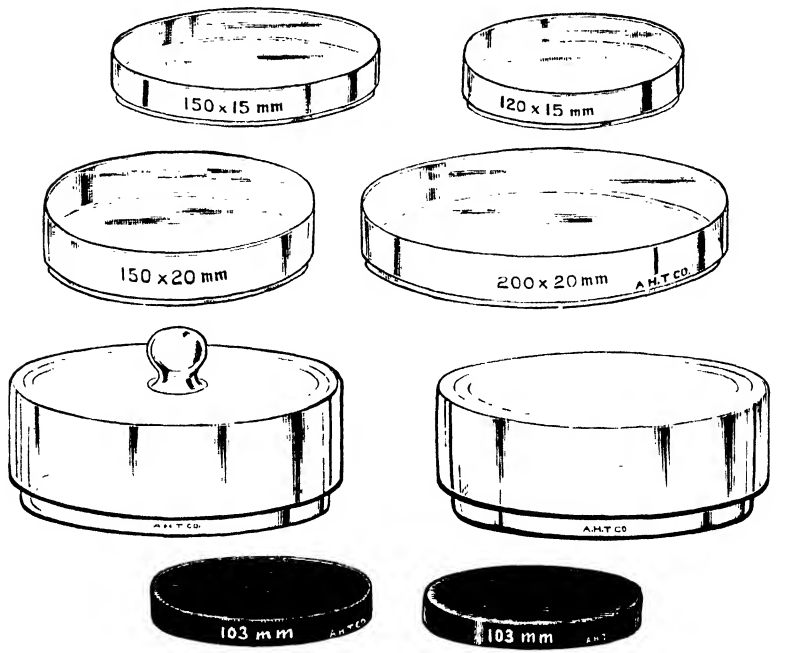


FIG. 2.—Showing Different Types of Culture and Petri Dishes for Microbiological Work.

The larger sizes are especially useful for culturing molds and other thread fungi.

The two illustrations at the bottom are porous covers for Petri dishes. They are used to absorb moisture and thus prevent spreading colonies.

prevent the entrance of extraneous organisms in the plates. The outgrowth of the need for a dish which would prevent contamination during incubation is the modern Petri dish, sometimes spoken of as a plate, and the process of making a Petri-dish preparation is often spoken of as " plating " or " plating out."

Petri dishes are available in several different sizes. Those which are 60 cm. in diameter and 1.5 cm. in height are perhaps

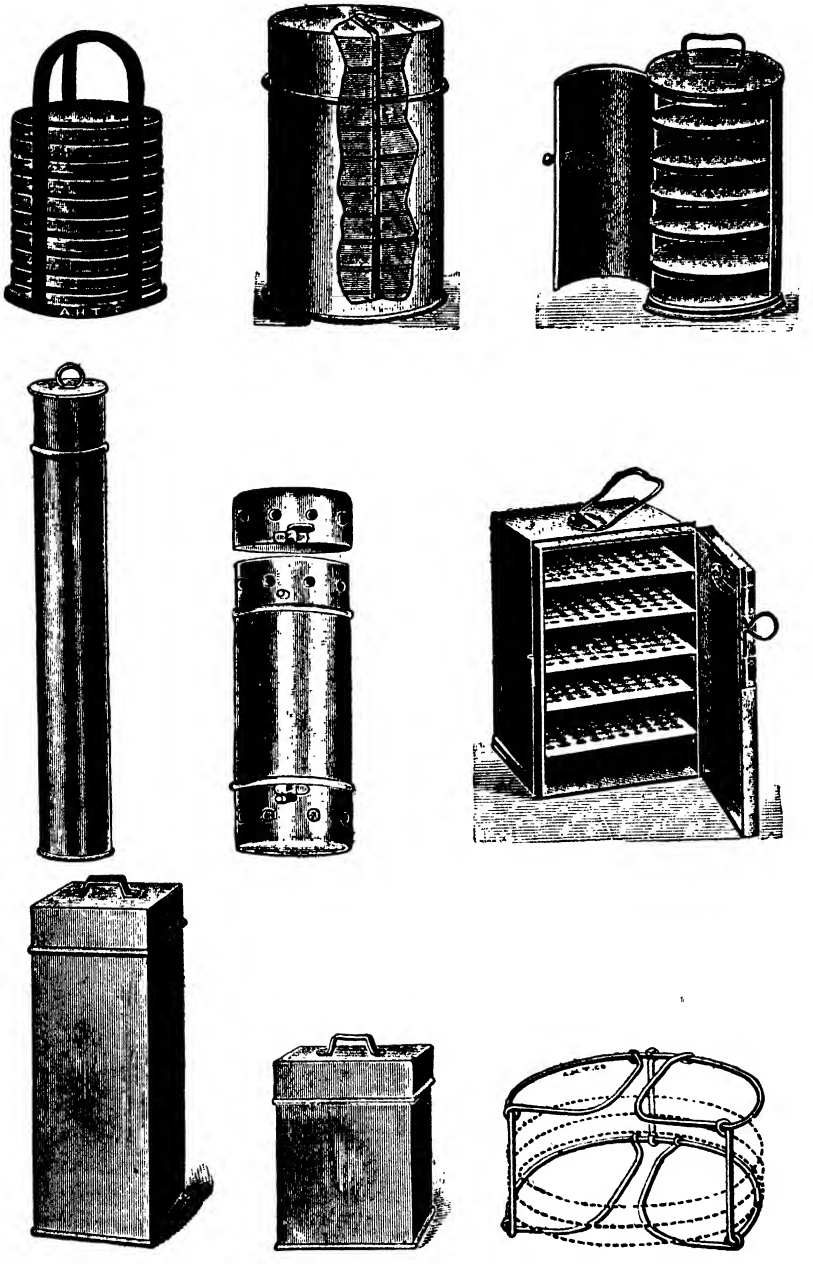


FIG. 3.—Types of Containers and Holders for Sterilizing Petri Dishes, Pipettes and Other Apparatus.

more satisfactory than other sizes. For special technics, such as certain of the anaerobic procedures, a larger size is convenient. In selecting Petri dishes, only those should be purchased that possess flat bottoms. Quite often the centers of the lower halves are found to be raised. This causes the medium to flow around the edges, giving a very uneven distribution of bacteria. It is also best to adopt one or two sizes for a laboratory in order not to waste time matching covers and bottoms, as is necessary when various sizes of Petri dishes are used.

Excessive moisture on the cover of Petri-dish cultures often causes spreading colonies and attempt must be made to avoid them. The agar plate cultures should always be inverted, thus putting the agar containing the organisms above the water of condensation. Very often even this is quite insufficient and other methods have to be resorted to. Hill (1904) used a cover made of porous earthenware rather than glass for the Petri dish, and thus got rid of the excessive moisture by absorption and evaporation. Some laboratories use the porous cover glazed on the outside, giving a firm surface for marking numbers, etc. In ordinary work such covers are not necessary, although it is wise to see them and thus know that they are available.

Dilution Bottles and "Water Blanks."—A "water blank" is a sterilized bottle containing a known amount of sterilized water. Water blanks are filled before sterilization, plugged with cotton and sterilized. Frost has shown that during sterilization the volume of water in dilution bottles may be decreased; this might cause considerable error where accuracy is desired. Noyes (1918) also studied the effect of sterilization in the autoclav on measured amounts of water in cotton-stoppered bottles. There seemed to be an average loss of 3.2 per cent of water from bottles with cotton stoppers. Dearstyne (1918) studied the effect of cotton stoppers used in dilution blanks on the bacterial count. Bacteria (*B. subtilis*) seemed to be unable to withstand sterilization temperatures when inside cotton stoppers. This author advises the use of glass-stoppered bottles, however, for water blanks. The container used depends on several factors. At times test tubes may be used when small amounts of dilution water are needed.

To avoid errors which may arise from the sterilization of known amounts of water, larger quantities of water may be sterilized and the desired amount transferred to the sterile bottle by means

of a sterile pipette. Thus the laboratory worker may be certain of the amount of water contained in the water blanks and may secure accurate quantitative data. Large transfer pipettes may

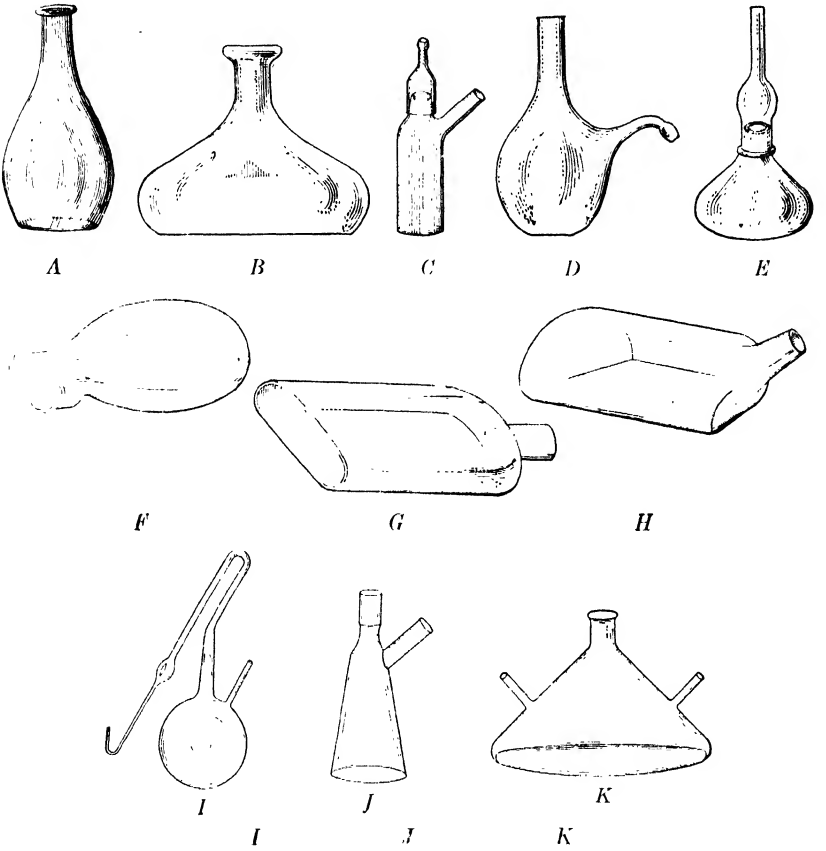


FIG. 1.—Types of Flasks for Bacteriological Work.

A, Koch's Culture Flask; *B*, Fernbach's Antitoxin Flask; *C*, Freudenreich's; *D*, Lister Culture Flask; *E*, Miquel Flask; *F*, Kolle Culture for Growing Bacteria in Large Numbers; *G*, Roux Flask; *H*, Piorowski's Flask; *I*, Pasteur's; *J*, Yeast Culture Flask; *K*, Fernbach's Flask with Side Tubes

be used for this purpose; they may be wrapped in paper before sterilization.

Fermentation Tubes. Several types of fermentation tubes are available, any of which may be used provided they hold a

sufficient excess of the medium over the volume of sample to be added. "Standard Methods for the Examination of Water and Sewage," 1917, recommends that the fermentation tubes used in water analysis should hold four times as much medium as sample.

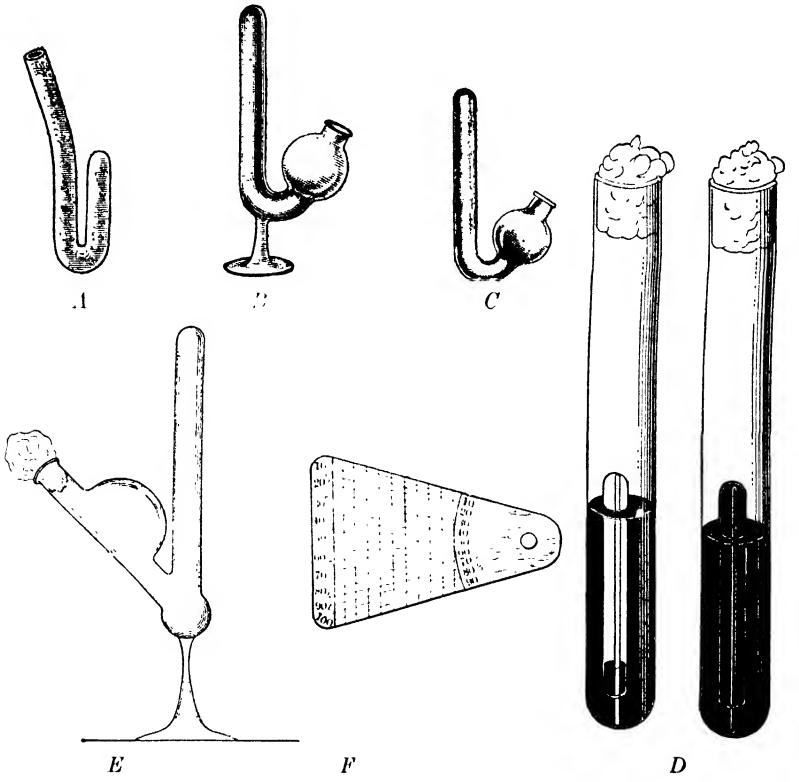


FIG. 5.—Several Types of Fermentation Tubes.

A, Brown's; *B*, Smith's with Foot; *C*, Smith's without Foot; *D*, Graves' Modification of the Durham Fermentation Tube; *E*, Fuller's Modification of the Smith Fermentation Tube; *F*, Frost Gasometer Chart for Reading Percentages of Hydrogen and Carbon Dioxide.

All fermentation tubes are constructed with a closed arm which retains the gases that are given off in bacterial metabolism. There are three general types now in use by bacteriologists, Brown's, Smith's (1890), and Durham's (1898).

The Brown fermentation tube was devised for use in water analysis and is quite similar to Smith's. The Smith tubes are

made both with and without glass feet. Those without the glass feet require a special rack but have the advantage, when placed in such a rack, that they do not fall over so easily. A convenient rack for holding Smith tubes has been devised by Bain (Illinois State Water Survey Bull. 6, 31, 1908). Another device for holding Smith fermentation tubes has been devised by Stull.

Frequent reports from various laboratories have indicated that the Durham type of fermentation tube is superior to the Smith tube. The advantages are apparent to anyone who has carried out many fermentation tests. There is little question

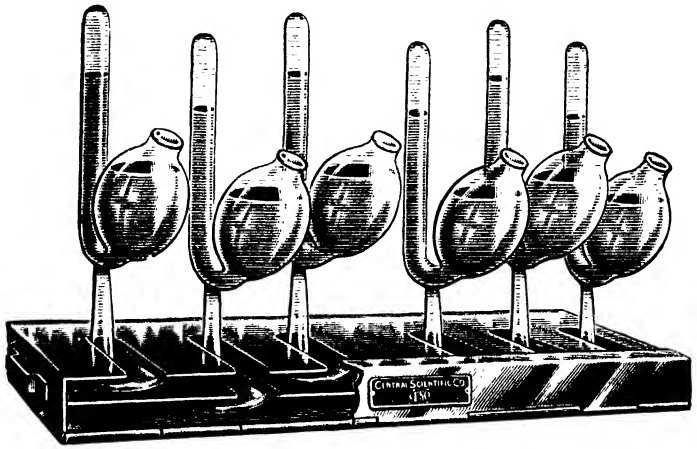


FIG. 6.—Stull's Fermentation Tube Support.
This is one of a number of types which are available for the Smith type of tube with foot.

that the Durham type of fermentation tube, made from an ordinary test tube and an inverted vial, is more convenient to handle and use in ordinary routine bacteriological work. No portion is made of thin glass as in the Smith type. Neither is a special apparatus required for holding it, for any test-tube rack suffices. There is one objection, however, that the inverted vial may rest so firmly on the bottom of the outer tube or " open arm " that the bacteria will find it difficult to enter. To avoid this, Lee and Fegeley (1914) proposed that the inner vial, the " closed arm," be cut off at an angle in order to make possible more ready ingress of motile bacteria. Graves (1917) accomplished the same ends by inverting the closed arm over a wooden rod which lifted the vial from the bottom of the open arm.

Efficiency studies have been made on both the Smith type and the Durham type of fermentation tube. Browne (1913) reported that the latter type was more efficient for low dilutions than the Smith type. In the higher dilutions, the Smith type was more efficient. Browne and Ackman (1917) and Browne (1917) have stated that the following factors cause variations in the amounts of gas produced in lactose peptone bile and lactose broth: (1) temperature; (2) time of incubation; (3) initial reaction of the culture medium; (4) length of inverted vial; (5) source of bile; (6) absorption of formed gas.

Another convenient method of following the fermentation of carbohydrates by a bacterium was announced by J. H. Brown. It has the advantage that a special tube is not needed. An ordinary culture tube is filled, as usual, with the carbohydrate medium and is overlaid with a layer of paraffin. After the tube has been inoculated and gas has been formed, the layer of paraffin is pushed upward in the culture tube. Oftentimes a vigorous fermenting microorganism will force the plug out of the culture tube.[1]

Baskets and Racks.—These are convenient articles in the bacteriological laboratory and are used in many different sizes and shapes. They are used for holding test tubes and for protecting other glass apparatus during incubation. Baskets may be secured in many different shapes and sizes from supply houses, or they may be made at any tin shop. It is probably best to secure only such baskets as are fastened together by wire, for those that are held together with solder will fall apart if, by chance, one is placed in the hot-air sterilizer.

Slides and Cover Glasses.—These should be made of clear glass and should be of uniform thickness. The cover glasses may be either round or square. Those 18 mm. square are best. The following data are of value in this connection.

THICKNESS OF COVER GLASSES

No. 1.—Varying from 0.13 to 0.17 mm. ($\frac{1}{200}$ to $\frac{1}{150}$ inch).
No. 2.—Varying from 0.17 to 0.25 mm. ($\frac{1}{150}$ to $\frac{1}{100}$ inch).
No. 3.—Varying from 0.25 to 0.50 mm. ($\frac{1}{100}$ to $\frac{1}{50}$ inch).

Number of Cover Glasses in ½ oz. Boxes

½ oz. box of No. 1, 18 mm. square contains 136 covers.
½ oz. box of No. 2, 22 mm. circles contains 90 covers.
½ oz. box of No. 2, 22×440 rectangles contains 156 covers.

[1] Brown, J. H., Jour. Exper. Med. 35 (1922) 667.

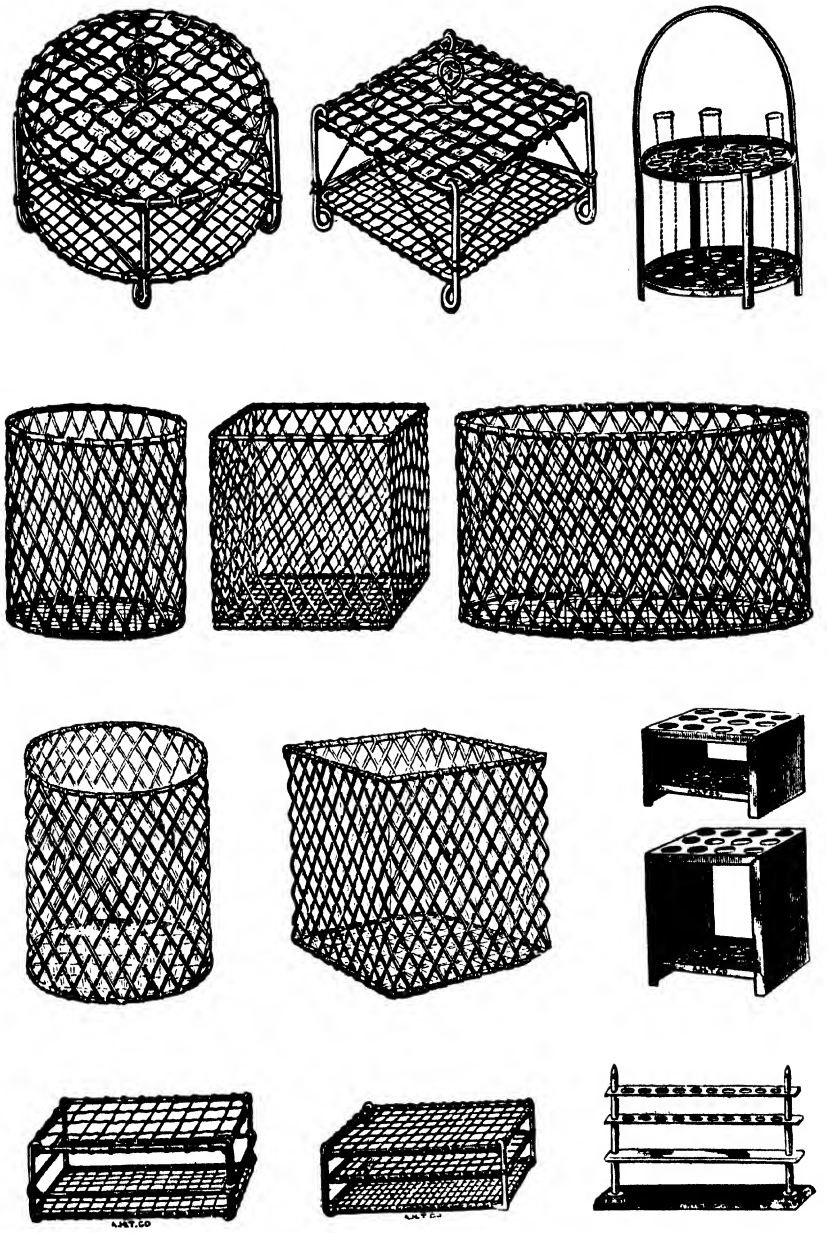


FIG. 7.—Various Types of Wire Baskets for the Bacteriological Laboratory.

It is absolutely essential that both slides and cover glasses be clean. They should be free from all traces of grease and other foreign matter. To accomplish this they should be boiled

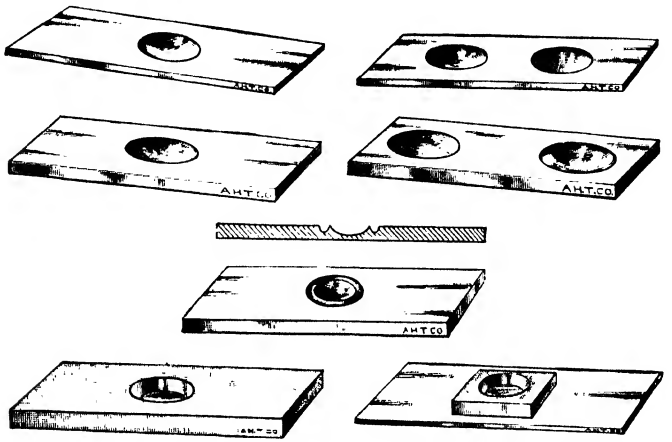


FIG. 8.—Showing Different Types of Slides which May be Used in Microbiological Work.

These slides may be used for hanging drops or moist chambers for the observation and culture of bacteria. (See also FIG. 33.)

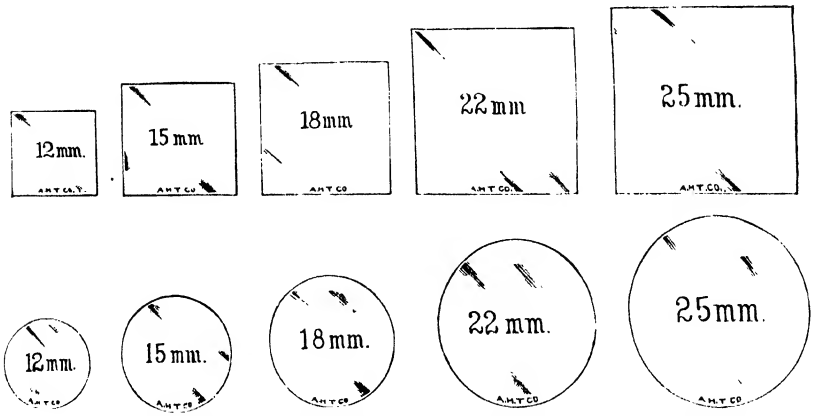


FIG. 9.—Showing Actual Sizes of Cover Glasses for Microscopical Work.

in cleaning mixture and thoroughly rinsed in tap and distilled water. After this they may be dried or stored for use in alcohol or ether. The reasons for using cover glasses of certain thicknesses are given in the chapter on the microscope.

Needles and Loops.—Platinum needles have extended use in the microbiological laboratory. While different sizes may be used, the best is, perhaps, gauge No. 26 or the two contiguous sizes. The lighter wires may be too flexible, while the heavier may require too long for cooling after they have been flamed.

Loops are used for transferring small amounts of growth or media in which growth has taken place, from tube to tube. In some cases these loops have been standardized to carry a certain amount of fluid; these are spoken of as standard loops. Instances of the use of such a loop are the standardization of disinfectants and the determination of the number of bacteria in feces according

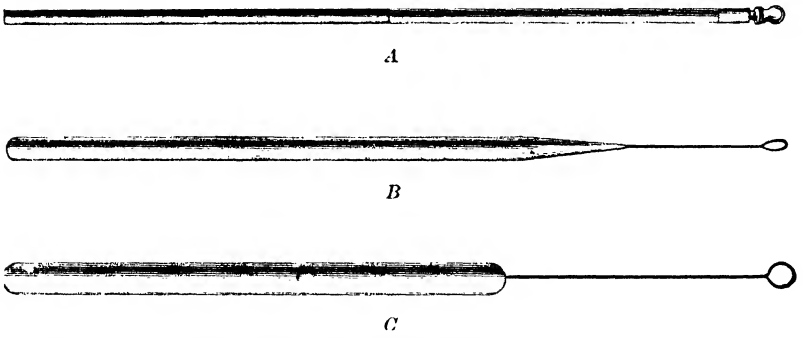


FIG. 10.—Types of Holders for Inoculating Needles and Loops.

A, Special Aluminum and Wooden Handle; *B*, Aluminum Rod; *C*, Glass Rod with Fused Wire

to the Eberle-Klein method. St. John (1914) devised a loop which gave drops of from 0.019 to 0.021 gram according to the angle at which it was taken from the fluid. Under more favorable circumstances the variation was from 0.017 to 0.023 gram. The size of the drop carried by this loop is about 0.020 c.c. Miller (1924), who studied standard loops, suggested that they could take the place of the pipette in milk analysis. He observed a satisfactory consistency between counts obtained by plating standard loops of milk and the counts secured when the pipette was used for making the standard agar plate. These data stimulated Miller to make a careful study of the possibility of perfecting a loop which could be used in quantitative work. For uniform work he made the following suggestions:

"1. The loop should be well made, round and smoothly soldered at the junction point or, even better, electrically welded.

"2. The loop should be held with the plane of the loop in a vertical position and it should be submerged in the liquid only enough to fully cover the loop.

"3. The loop should be withdrawn from the fluid slowly. As the loop comes out of the fluid, the fluid draws downward under the combined influence of gravity, surface tension, and viscosity, and tends to assume a definite biconcave shape. The loop should be withdrawn so slowly that these forces are at all times in equilibrium, and especially at the end when the pulled-up portion of the surface which adheres to the loop finally snaps off and the loop is free. This slow removal of the loop from the surface of the liquid is essential if uniform results are to be obtained."

Miller determined the capacity of his loops by weighing the amount of liquid delivered. This was done by weighing about twenty loopfuls rather than one.

The high cost of platinum has forced bacteriologists to use substitutes. Nichrome wire is suitable for needles and loops.

Pipettes.—Many different types of pipettes are available. Some of those used by bacteriologists are especially made. Good bacteriological pipettes should have the following characteristics:

1. Should be of uniform size both as to diameter and length.
2. Should have uniform internal diameter and even aperture.
3. The distances between graduation marks must be uniform.
4. The distance between the zero point (top of scale) and the top of the pipette should be at least 20 cm.
5. The distance between the lowest graduation mark and the tip of the pipette should be at least 4 cm.

Two types of pipettes, called, respectively, "transfer pipettes" and "measuring pipettes," are used by bacteriologists. The transfer pipettes are graduated to but one mark and will deliver only a known amount of fluid. The "measuring pipettes" are graduated between the two limiting marks so that amounts of liquid between these points may also be delivered.

Pipettes are also certified to deliver amounts of fluid between certain limits of error. Some are unofficially certified by the manufacturers while others are officially certified by the Bureau of Standards. The latter are supplied with Bureau of Standards certificates and represent the most reliable pipette available. For ordinary work, such pipettes are not necessary.

Instances of infection by handling disease bacteria in pipettes are by no means unknown. In order to render this less possible

the tips of the pipettes may be plugged with cotton which is pushed down sufficiently to cause no difficulty when the finger is put over the end. The very fact that there is an alternation between the use of the mouth and hand in transferring liquids in pipettes makes the ordinary ones dangerous. When materials or cultures that are especially dangerous have to be handled in pipettes, rubber bulbs may be placed over the upper end. These make it unnecessary to apply the mouth to the pipette.

Plugging Tubes, Flasks, etc.—Bacteriologists find plugs made from cotton more satisfactory than corks. It is necessary to have some ventilation in culture tubes and flasks so that gases formed by the microorganisms may pass out and that air may enter. It is also necessary to have a closure which will fit the container tightly and withstand sterilization at high temperatures. Cotton has been found to be the best and consequently it has been universally adopted. It strains out other bacteria which might work themselves into a container about a cork stopper.

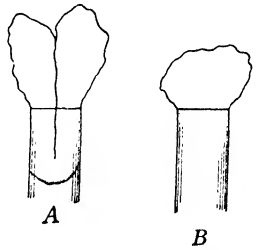


FIG. 11.—Showing Different Types of Cotton Plugs.

A, An Undesirable Plug. Such a plug will spring apart when removed from the tube; *B*, A desirable rolled plug which not only protects the culture tube but which remains compact when removed from the tube

The making of plugs is a very important step in bacteriological procedure and one that may often be neglected. For most careful work, an extra-good grade of cotton, such as absorbent cotton, should be used. This ordinarily contains little lint and consists of clean, straight fibers. For ordinary work the cotton in bats is satisfactory. Some brands are more wiry than others, yielding plugs which will not hold their shape but will unroll when pressure is released. Rapid rolling of good cotton plugs results only from much practice.

Rolled Plugs.—Rolled plugs require more time but are most satisfactory for containers that are to be kept under observation for some time and from which the plug will be removed and replaced frequently. These plugs retain their shape better than stab plugs. They may be made by taking a piece of cotton about 3 inches square and folding in the sides, giving a rectangular piece of cotton the width of which is determined by the desired length of the plug. The plug is then completed by rolling the cotton

from one end. The plug should be rolled and not twisted. This will give a plug the ends of which are clean, with very few extending fibers. It will be solid and, after a little experience, can be made to fit the container nicely.

Stab Plugs.—The "stab" plug (so called for the lack of a better name) requires much less time and practice. A piece of cotton, of sufficient size to fit the mouth of the container, is placed over it and pushed into it by means of a nail or lead pencil. This yields a plug with much cotton protruding from the tube. Such plugs are difficult to handle, inconvenient to flame, and oftentimes shapeless when withdrawn from the mouth of the container and replaced. Stab plugs are probably sufficiently satisfactory for containers that are to be used but once. However, rolled plugs are worth all the trouble it takes to make them, especially if they must be removed and replaced a number of times.

Incubators.—An incubator is an apparatus constructed for the maintenance of a constant temperature and humidity. Several different contrivances have been developed, varying from those of simple construction to those that are intricate. Some are heated by gas, others by electric lamps and others by automatic electric heaters. Most of them have an automatic control which, after adjustment, should keep the temperature close to that desired. It is impossible, of course, to have all locations in the incubator at exactly the same temperature. Those closest to the heaters will be warmer. Some incubators are provided with fans for stirring the air. The air should also be kept moist, else prolonged incubation of culture media will result in evaporation.

It is impossible to keep incubators exactly at one temperature unless delicate control devices are provided. These may be too expensive for ordinary installations. Consequently, a degree of variation on both sides of the desired temperature is usually allowed.

Besides these larger incubators, another type may be used for incubation on the stage of the microscope. These are called "warm stages" and may be electrically heated and regulated. They are small contrivances, very much like the moist chamber which has become an important adjunct to laboratory technic. Illustrations of "warm stages" may be seen in catalogues of the several supply houses.

Miscellaneous Apparatus.—Besides the apparatus just discussed, which even the introductory student must use, or, at least, should know about, there are a great number of pieces that are used only by the advanced student.

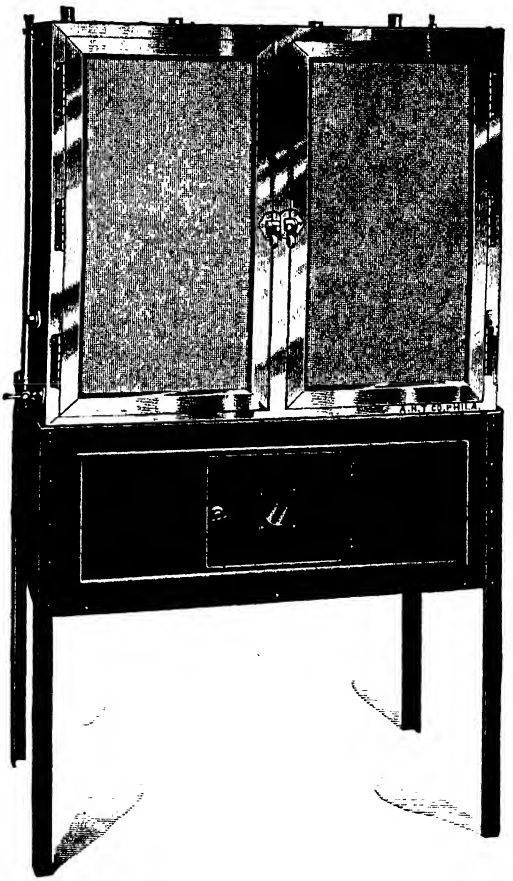


Fig. 12.—Showing Incubator (Hearson Type) for Microbiological work.

This is one of several models and sizes.

Sterilization and Disinfection

Bacteria do not ordinarily exist in nature as pure cultures. Since there are so many varieties, the bacteriologist must use media and apparatus which are free from not only bacteria but all

forms of life. This condition is accomplished by sterilization, which is to be distinguished from disinfection—the removal of pathogenic bacteria only. The commoner methods for sterilization may be classed as follows:

I. Dry Heat.
   a. Flaming and incineration.
   b. Hot-air oven.

II. Moist Heat.
   a. Inspissator.
   b. Low-pressure steam in Arnold steam sterilizer.
      1. Continuous method.
      2. Intermittent method.
   c. High-pressure steam.
   d. Boiling.

III. Filtration.
   a. Through liquids.
      (Sodium hydroxide, sulfuric acid, etc.)
   b. Through solids.
      (Sand, glass wool, cotton, porcelain, etc.)

IV. Light.
   a. Sunlight.
   b. Ultraviolet light.

DRY HEAT

Death by drying may be the result of two processes: the cell protoplasm may decompose, or oxidation may take place. This has been studied by Paul (1909) and his co-workers who found, in general, that the rate of death in dry heat was proportional to the oxygen concentration. This would seem to indicate that death in dry heat was an oxidation process, although other reactions may enter. With regard to moist bacteria, the oxidation processes would then be limited by the solubility of the oxygen in water. According to Winkler (1889), this is 10.14 parts per million at 15° C. and 760 mm.

Incineration.—As a means of sterilization, this method needs no discussion. It is plainly a process of oxidation and is very efficient. Small incinerators which will handle practically all ordinary material may be purchased and should be available in laboratories.

Flaming.—This simple method is a serviceable one to the bacteriologist. Platinum needles are sterilized by this method. Watch glasses, slides, etc., may be completely sterilized if a little care is used during the flaming process. The Bunsen burner or alcohol lamp may furnish the heat. The sterilization of the platinum loop and needle by this method should be done with caution. If the needle carries considerable growth, this may sputter and fly out of the flame. The needle should be introduced into the flame quite slowly and held there until it has turned to a deep red. It is also a good thing to flame the lower portion of the handle of the needle in order that any microorganisms thereon may be destroyed. This part of the handle usually enters the culture tube or other container.

Hill (1900) recently described an adaptation of the incidental method of sterilization which involved dipping the article to be sterilized into methyl alcohol and burning. Sanderson (1922) called attention to the possibility that bacteria may pass through unharmed. The time required for the alcohol to burn off was said to be too short, especially if spore-forming bacteria were present. Sanderson arrived at the conclusion that Hill's method does not always give conditions of sterility.

Hot-air Sterilization.—Hot-air sterilization requires the use of an oven with an adequate supply of heat. In well-equipped laboratories gas is usually used although any source of heat could be utilized. Hot-air sterilization is usually applied to dry apparatus since the temperature required is much above the boiling point. The time and temperature of operation varies; however, there are certain minimum limits below which this process should not be conducted. The reasons for this may be inferred from data showing the effect of moisture content on the temperature of coagulation of proteins. Frost and McCampbell reported that egg white with no added water coagulated at 160° C. In the hot-air oven we should expect a minimum concentration of water and therefore should realize that temperatures above 160° C. would have to be used.

Precautions for Use of Hot-air Sterilizer

1. Only dry apparatus should be put in.
2. The temperature should be raised slowly to permit slow, even expansion of glass.
3. The sterilizer should not be opened until the temperature has lowered. Cold air will cause the hot apparatus to crack.

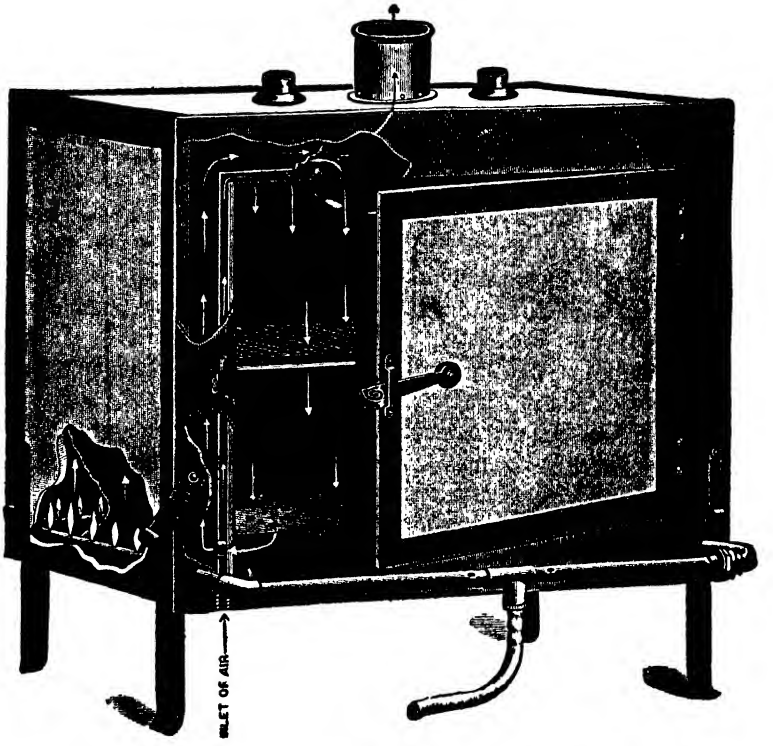


Fig. 13.—Hot-air Sterilizer, Lautenschlager Type.

MOIST HEAT

The theory of sterilization by moist heat has been studied by Chick (1910) and others. Here three essentially different processes may take place:

1. Direct effect of heat on bacterial protein.
2. Effect of water, possibly hydrolytic, on these proteins at high temperature.
3. Desiccation of bacteria.

It was pointed out by Miss Chick that an analogy exists between the disinfection of bacteria by hot water and the " heat coagulation " of proteins. This may help to account for the difficulty with which spores are killed by hot water when compared with the vegetative cells. The protein in the spores may be more resistant to hydrolysis than that in vegetative cells. Both changes follow the monomolecular law and both are greatly increased by the presence of minute amounts of acid. Chick (1910) stated that the striking similarity between the effects of temperature (dry) on the one hand and hot water on the other indicate that disinfection by the latter is due to the action of water (coagulation and alteration) upon some one protein which is essential for life of the bacterium, and that the reaction is conditioned by the chemical action of water upon its constituent proteins. The following facts with regard to disinfection by moist heat were established by Chick:

1. Disinfection proceeds according to the logarithmic law, the rate of disinfection being proportional, at any moment, to the concentration of the surviving bacteria.

2. The presence of minute quantities of acid or alkali, too small to produce any direct disinfectant action, increases the reaction greatly. The acid, however, gives the greatest increase. This presents close analogy to the " heat coagulation " of proteins.

Rubner (1913) reviewed the application of steam to sterilization. He pointed out that vegetative cells contain much more water than spores and thus succumb more quickly to the action of heat. Spores contain hygroscopic water which soon evaporates, leaving dry bacterial protein. This will resist coagulation for some time. Steam at 100° C. is an important chemical agent since hydrogen sulfide, ammonia and carbon dioxide are liberated from keratin, casein and dried bacteria when they are subjected to its influence. Rubner found the saturation of the steam to be about as important as the temperature. Steam at 100° C. with a saturation of 80 per cent required five times as long to kill bacteria as did saturated steam, and steam with a saturation of 70 per cent required twenty-two times as long as saturated steam. If steam at 100° C. is superheated, it is altered in two ways. The temperature is raised, which makes it a more powerful disinfectant, and the saturation is lowered, which makes it a weaker disinfectant. In superheated steam at 110° C., made from steam at 100° C.,

spores lived twice as long, and at 127° C. ten times as long. This suggests the correlation between the temperature and saturation which are very important factors in sterilization. According to the laws of disinfection, time is the other factor and it cannot be separated from the first two.

High-pressure Steam.—This method has extensive use in bacteriology. The apparatus involved is called an autoclav or dressing sterilizer. Several types are used, the most convenient of which are connected to a steam main from a power plant. Where this is impossible, it may be necessary to generate the steam under the sterilizer. This is less convenient since it takes some time to raise the pressure. Often it is necessary to keep water in the bottom of the sterilizer in order to prevent superheating of the steam. Under practical conditions this precaution may usually be ignored since the steam will be moist enough. The following table shows the relation between ordinary pressures and temperatures required for sterilization in the autoclav:

Gauge Pressure	Temperature, Centigrade	Gauge Pressure	Temperature, Centigrade
0	100	15	121.5
5	109	20	126
10	115.5	40	141

In the presence of water, Chick has shown that the temperature is an important factor in sterilization. The temperature coefficient was determined for *Eberthella typhi* (*Bacterium typhosum*) and was reported to be 1.635 per 1° C., or 136 per 10° C. Here again the killing of bacteria in the presence of water was found to be analogous to the "heat coagulation" of their constituent proteins.

The autoclav may be used for sterilizing many pieces of apparatus and many media. It has the advantage, over other methods, of taking less time. It has been recently shown that less hydrolysis of polysaccharides is secured in the autoclav than in the Arnold. This is contrary to what was once believed, for it is stated in many places that the Arnold steam sterilizer should be used for such substances as complex sugars, to prevent hydrolysis.

Boiling.—Practically no special apparatus is required for this method, and any that is demanded may be quickly secured. Probably fifteen minutes is sufficient for vegetative cells but spores are too resistant to be killed so easily. Surgical instruments may be sterilized by boiling. The water should be boiled for ten minutes before the instruments are put in, in order to prevent the possibility of rusting or harming the cutting edges. Boiling is a useful method of sterilization for the home as well as the laboratory.

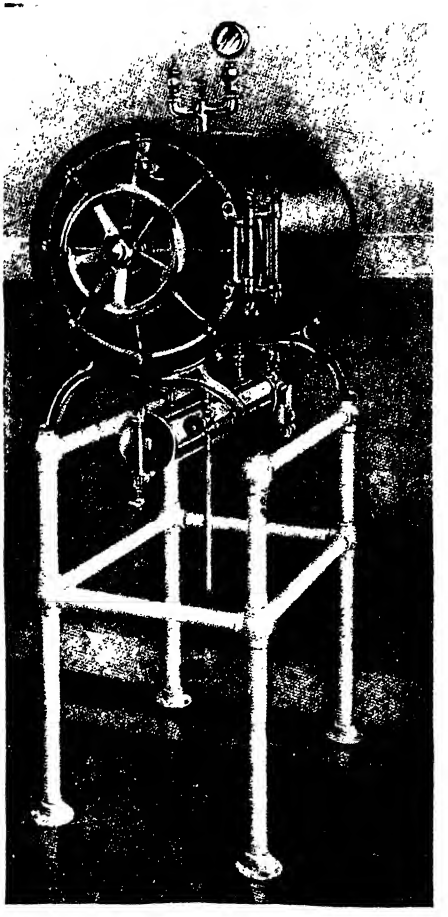


FIG. 14.—Autoclave or Dressing Sterilizer.

Streaming Steam (Free-flowing Steam).—The apparatus which is used in this method is much like the ordinary steamer used in the kitchen. In the bacteriology laboratory the modification of this apparatus is known as an Arnold steam sterilizer. It consists essentially of a metal box with a false bottom through which the steam rises to escape at the top. There can be no superheating in the Arnold, as is possible with the autoclav, and it requires less attention. It may be used in two ways—continuously or intermittently.

In the continuous method the material to be sterilized is heated for from thirty minutes to an hour and a half. This method has the disadvantage, however, that such prolonged heating may cause changes in the materials. Di-, tri-, and poly-saccharides

may be hydrolyzed, proteins coagulated, etc. Such has been found to be the case, as mentioned elsewhere.

In the intermittent method the material is heated for twenty to thirty minutes on each of three successive days. On the first day the vegetative cells are killed. The second day's heating will destroy those vegetative cells which have come from spores that survived the first day's heating. On the third day sterilization is simply checked up and made certain.

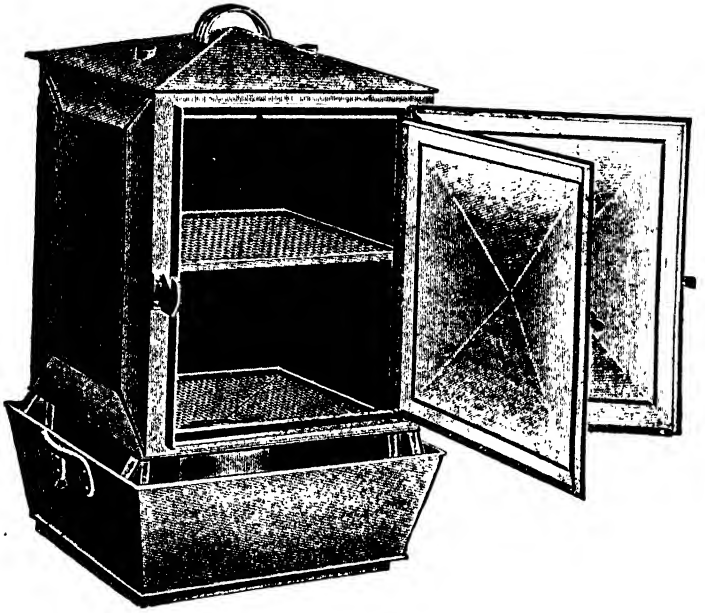


FIG. 15.—Arnold Steam Sterilizer. (Boston Board of Health Pattern.)

Inspissator.—Some media which are used by bacteriologists must be sterilized at low temperatures. For this purpose a serum coagulator, or inspissator, is used. The media are heated at from 57° to 60° C. for different lengths of time. Blood serum may be heated for periods of one hour or for a longer period at one time.

STERILIZATION BY FILTRATION

Filtration is an efficient method of sterilization and has certain distinct advantages over other methods. It leaves the filtrate

sterile and unchanged in chemical constitution. Body extracts and sera may be thus treated to render them sterile.

Filtration through Liquids.—The action of filters in sterilization is entirely mechanical unless some strong chemical, such as sodium hydroxide or sulfuric acid, is used. In such cases the bacterial substance is destroyed by chemical action. Filtration

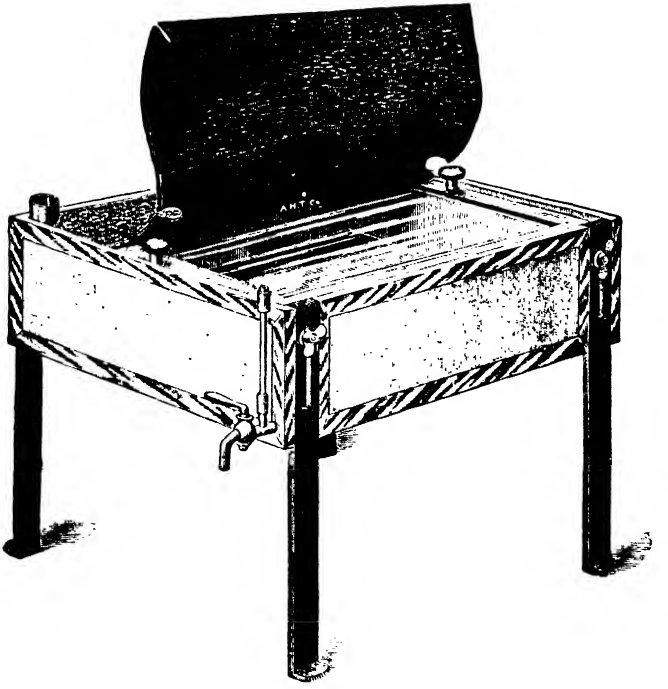


FIG. 16.—Bath for Sterilization at Low Temperatures (Inspissator).

methods are used to strain the bacteria out of air in order to ascertain its bacterial content.

Filtration through Solids.—The same principles are involved here as in the use of cotton plugs in culture tubes and flasks. While different substances have been used, most attention has been given to cotton, wool, glass wool, sand, etc. Filters especially adapted to laboratory use in bacteriology are made from diatomaceous earth, clay, etc. They are available in different sizes and are easily cleaned and sterilized. Such filters must be made with great care, for the bacteriologist must have assurance

that the pores are small enough to hold back the smallest bacteria; if they were not, some cells would pass through and seed the filtrate with living organisms.

Dialysis.—This method of sterilization by filtration is not used much in practice. It is used in experimental work where it is desired to have one species subjected to the metabolic products of another without having the different species in immediate contact.

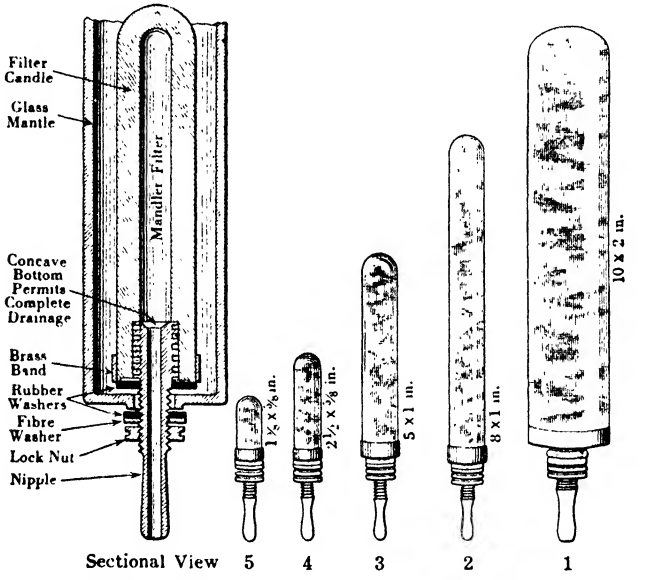


FIG. 17.—Mandler Filters for Bacteriological Work.

Preparation and Storage of Sterile Apparatus.—Bacteriologists find it convenient and necessary to keep a supply of sterile apparatus on hand. The smaller pieces, such as small culture tubes, watch glasses, etc., may be cleaned and sterilized in larger containers. Such cases for holding pipettes have been shown in Fig. 3. If such containers are not available, such apparatus as Petri dishes and pipettes may be wrapped in paper before sterilization and stored in this manner after sterilization. Empty apparatus should be thoroughly cleaned and plugged with cotton before sterilization. If it is to be kept for an indefinite period after sterilization, it should be wrapped in paper and stored in a

cabinet in which no other apparatus but that which has been sterilized is stored.

BIBLIOGRAPHY

BAILLIE, W. L., and WILSON, F. E. An Autoclav Test for the Grading of Chemical Glassware. Jour. Soc. Chem. Ind. 41 (1922), 45-56.

COLLINS, W. D. Report on Standardization of Sizes and Shapes of Apparatus. I. Jour. Ind. Eng. Chem. 13 (1921), 1070.

COLLINS, W. D. Report of Committee on Guaranteed Reagents and Standard Apparatus. Jour. Ind. Eng. Chem. 13 (1921), 402.

COLLINS. W. D. Laboratory Thermometers. Jour. Ind. Eng. Chem. 13 (1921), 240.

COLLINS, W. D. Report on Standardization of Sizes and Shapes of Apparatus. II. Jour. Ind. Eng. Chem. 14 (1922), 654-659.

ESTY, J. R., and CATHCART, P. H. The Change in the Hydrogen Ion Concentration of Various Mediums during Heating in Soft and Pyrex Glass Tubes. Jour. Inf. Dis., 29, 29-39.

FABIAN, F. W., and STULL, R. C. A Study of Hydrogen Ion Concentration of Different Kinds of Glassware when Sterilized with Buffered and Non-buffered Solutions. Absts. Bact., May, 1921, Abst. No. 1045. See also Report of the Bacteriological Section. Sixtieth Annual Report of the State Board of Agriculture.

Testing of Thermometers. Bureau of Standards. Circular No. 8, 1911.

Testing of Glass Volumetric Apparatus. Bureau of Standards. Circular No. 9.

WAIDNER, C. W., and MUELLER, E. F. Note on Partial and Total Immersion hermometers. Jour. Ind. Eng. Chem. 13 (1921), 237.

WALKER, P. H., and SMITHER, F W. Comparative Tests of Chemical Glassware. Tech. Paper. Bureau of Standards. No. 107 (1918).

CHAPTER II

THE MICROSCOPE

SOME knowledge of the construction of the microscope is quite necessary if the investigator is to secure the best results. The following paragraphs have been prepared from several sources, including especially the little booklets published by the several manufacturers of microscopes. That prepared by the Bausch & Lomb Optical Company has been especially useful. These booklets should be reread occasionally by all who use the microscope. The microscope is a wonderful instrument both with respect to its mechanical and its optical features, especially the latter.[1]

General Information.—Before withdrawing the instrument from its case, note how it is accommodated therein. It should be carefully withdrawn and placed on the laboratory table for study and examination. It should be so placed that its use does not strain or fatigue any portion of the body. The heel of the instrument should be placed at the edge of the table or, if the instrument is to be inclined, a little distance in from the edge. When this has been done the instrument may be examined while reading with regard to its constitution progresses. It is better to have a microscope before you as you read in order that the names of the various parts may be more quickly acquired.

[1] Use and Care of the Microscope. Bausch & Lomb Optical Company, Rochester, N. Y.

How to Use and Care for the Microscope. Spencer Lens Company, Buffalo, N. Y.

The Microscope and Some Hints on How to Use It. E. Leitz, New York.

GAGE, S. H., The Microscope. The Comstock Publishing Co., Ithaca.

CARPENTER, W. B., The Microscope and Its Revelations. J. and A. Churchill, London, 1901.

GENERAL CONSTRUCTION OF THE MICROSCOPE

The illustration in Fig. 18 should be studied for the names

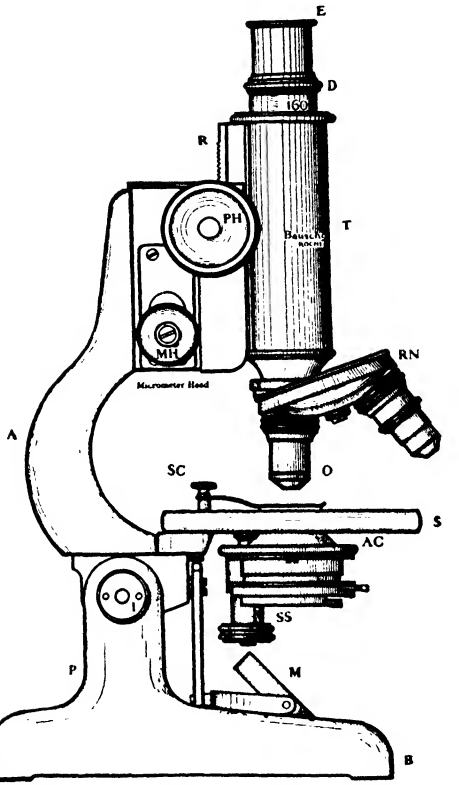


FIG. 18.—Names of Various Parts of a Microscope.

A	Arm	*O*	Objectives
AC	Abbé Condenser	*P*	Pillar
B	Base	*PH*	Pinion Head
D	Draw Tube	*R*	Rack
E	Eyepiece	*RN*	Revolving Nose
I	Inclination Joint	*S*	Stage
M	Mirror	*SC*	Spring Clip
MH	Micrometer Head	*SS*	Substage
		T	Body Tube

of the various parts of the instrument. These parts may be tabulated as follows:

A. MECHANICAL PARTS

1. The Body with Its Draw Tube.—The draw tube is provided with a millimeter scale which shows the total length of the working

tube reckoned from the shoulder of the objective screw to the eye-lens of the eyepiece. The reasons for this adjustable tube are discussed in the paragraph on objectives.

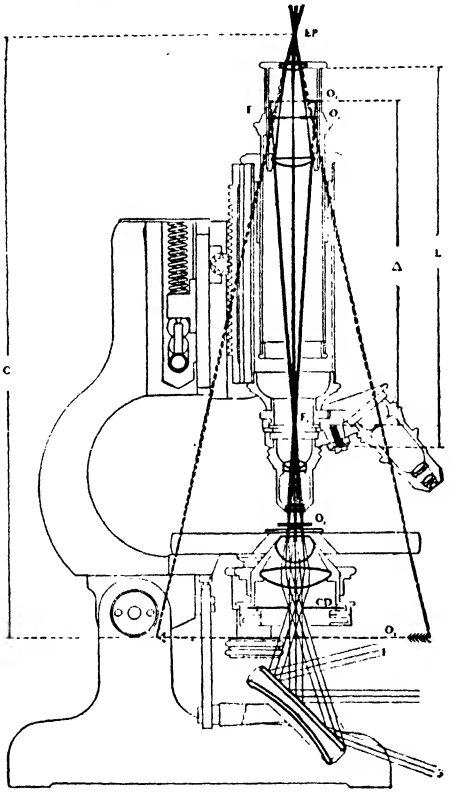


FIG. 19.—Index to Diagram.

F^1 Upper focal plane of objective.
F^2 Lower focal plane of eyepiece.
Δ Optical tube length = distance between F_1 and F_2.
O^1 Object.
O^2 Real image in F_2, transposed by the collective lens, to
O^3 Real image in eyepiece diaphragm.
O^4 Virtual image formed at the projection distance C, 250 mm. from
EP Eyepoint.
CD Condenser diaphragm.
L Mechanical tube length (160 mm.).
1,2,3 Three pencils of parallel light coming from different points of a distant illuminant for instance, a white cloud, which illuminate three different points of the object.

2. The Stage.—This is the part of the microscope on which the object to be examined is placed. It has an opening in the

center over which the object slide is placed and up through which the light comes to illuminate the object.

3. The Pillar.—This is the part of the microscope that supports the stage. At its upper part there is a hinge or inclination joint.

4. The Coarse Adjustment.—The rack and pinion is now universally used in place of the old sliding tube. This is a closely fitted system and must be kept clean.

" Care of the Coarse Adjustment.—Special care should be taken to keep the coarse adjustment free from dust, the effect of which is particularly pernicious. The slides and rack and pinion are necessarily exposed and the lubricant is likely to catch dust and also to gum. The tube should be occasionally withdrawn from the arm and the slides carefully wiped with a cloth moistened with Xylol. Lubricate by applying a small quantity of paraffin oil to a cloth and wiping well over the surfaces, removing the superfluous amount with a dry cloth. The teeth of neither rack nor pinion should ever be lubricated. An occasional cleaning of the teeth with an old tooth brush is advisable.

" It is advisable occasionally to lubricate the pinion shank on both sides of the arm with a very minute quantity of paraffin oil.

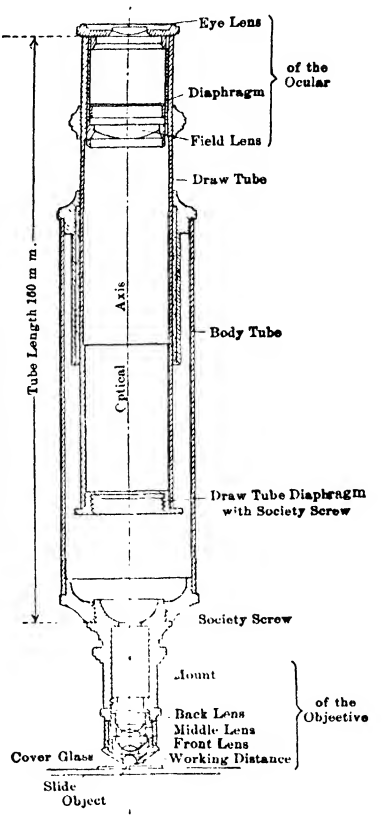


FIG. 20.—Showing a Cross-section of a Draw Tube from a Microscope. (After Spencer Lens Co.)

" *If the pinion works loose* from jar incident to transportation or long use, which sometimes occurs to such an extent that the body will not remain in position, increase the friction upon it by tightening the screws on the pinion cover."

5. The Fine Adjustment.—The fine adjustment has a limited range. The older types of microscopes have the micrometer head on the top of the arm or limb; the newer instruments have two micrometer heads, one on either side of the arm.

"**Fine Adjustment.**—In a general way it may be said that if the fine adjustment ceases to work satisfactorily the instrument had better be returned to the maker, as it involves the most delicate working and few people are conversant with its construction. There is very seldom any occasion for this, however, if used with reasonable care.

"*If the fine adjustment does not respond* to the turning of the micrometer screw, or if it comes to a stop, it indicates that the adjustment screw has come to the limit of its motion at either end. It should by no means be forced; it should at all times be kept at a medium point.

"**To Focus with Fine Adjustment.**—After the focus has been found with the coarse adjustment, the fine adjustment should be brought into action, in order to obtain a more sensitive and slower adjustment for focusing through the different planes or depths of the object. Its range of movement is necessarily short and at one end the screw comes to a stop, and at the other goes beyond the limit of movement and becomes inoperative. It should always be kept as near as possible *at the medium point of its range.* Grasp the milled head of the fine adjustment with the thumb and forefinger of one hand (right) and, turning the screw in either direction, focus in different planes of the object, while the other hand (left) moves the object."

6. The Substage Diaphragm.—

"*Use of Substage Diaphragm.*—The purpose of the diaphragm is to modify the amount of light, and by its aid obtain results in definition of the object which without it are impossible. Much will depend upon the density of the object, the intensity of illumination and the power of the objective.

"*Use an opening in the diaphragm of about the same size as the front lens of the objective.*

"As a rule this will be found to give a superabundance of light, especially in low-power objectives, and by reducing the aperture it will be found that there will be an increased differentiation in the object. The diaphragm should be reduced to a point where the amount of illumination will be perfectly comfortable to the eye.

"*Do not use so large an opening that there will be an uncomfortable glare, nor so small that undue exertion is required to see structure.*

"When oblique light is used, there should be no obstruction to the course of light, and with the iris or revolving diaphragms full opening should be employed."

"**Care of the Stand.**—'*Keep free from dust*' *is one of the first rules to be observed.* When not in use place the microscope in its case, or cover with a bell jar or close-mesh cloth such as cotton flannel or velvet, which should reach to the table. If dust settles on any part of the instrument remove it first with a camel's hair brush and then wipe carefully with a chamois skin, wiping with the grain of the finish of the metal and not across it, as in the latter case it is likely to cause scratches.

"*When handling the stand,* grasp it by the pillar or handle arm. While the arm is the most convenient part it is at the same time the most dangerous to the fine adjustment except in instruments of the handle-arm type.

"*Avoid sudden jars*, such as placing upon the table or into the case with force.

"*Remove any Canada balsam or cedar oil* which may adhere to any part of the stand with a cloth moistened with Xyol, and wipe dry with chamois.

"*Use no alcohol* on lacquered parts of the instrument as it will remove this finish. As the latter is for the purpose of preventing oxidization of the metals, it is important to observe this rule. Parts finished in black are usually alcohol-proof.

"*To use the draw-tube, impart the spiral motion.*

"*In using a screw driver* grind its two large surfaces so that they are parallel and not wedge-shaped, so it will exactly fit in the slot of the screw-head. Turn the screw with a slow steady motion, pressing the screw driver firmly into the slot. No screw-head will ever be injured if these points are observed."

B. Optical Parts

These, of course, are the most significant parts of the instrument. The mechanical parts have been improved solely to bring the optical parts into use more easily and accurately.

Magnification.—This may be determined by multiplying the objective magnification by the eyepiece magnification. The magnifying power of an objective is in inverse ratio to its focal distance. These are marked on these parts. At the end of this chapter are given magnification tables for several of the more common makes of microscopes. At first thought magnification would seem to be one of the most important ends desired in the use of a microscope. However, definition is perhaps equally important. As the magnification increases there may be a loss of definition and detail.

Optical Glass.—The glass used for the making of lenses is of a special kind which has required much experimental work to perfect. Before the World War most of the optical glass was made abroad, but after the beginning of the War most countries found it necessary to study its manufacture. To-day there are a number of places where a high-grade optical glass is being produced.

1. The Eyepieces.—These are also called oculars. They consist of a combination of lenses fitted into a short tube which drops into the top of the draw-tube. Oculars are designated usually by numbers, although letters may also be used. In general, the oculars with the lower magnifying power are designated with the lower numbers or letters.

Which Eye to Use.—The right eye is generally used for observations, but while the manipulator may from habit be inclined to use this, it may be

possible that in some cases the left can be used to best advantage and with less fatigue.

"*Make it a habit at the outset to keep both eyes open.*

" There is a point just above the eyepiece, called the *eye-point*, at which the rays cross within the smallest compass, and *this is the proper position for the eye, as the largest number of rays enter it.*

" *When above or below this point* the size of field will be reduced or shadows or colors will appear in it. In low-power eyepieces the eye-point is some dis-

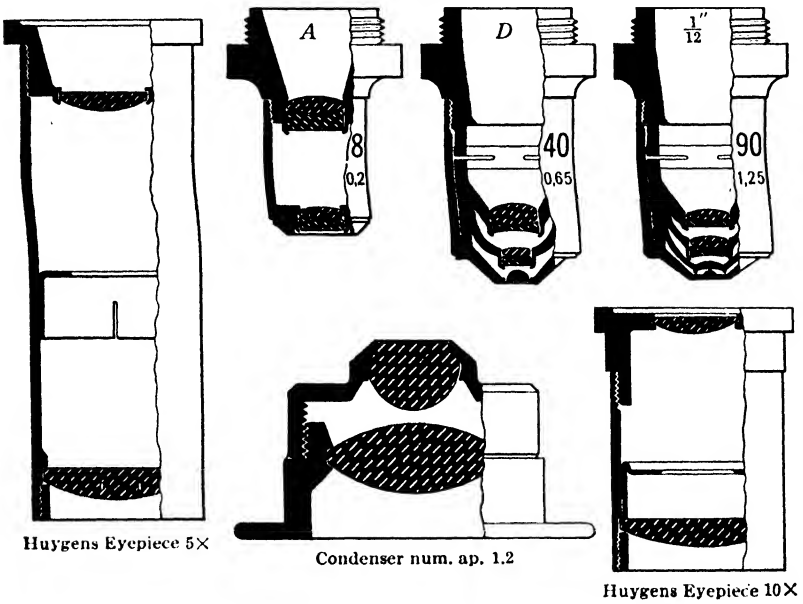


FIG. 21.—Showing Cross Sections of Various Parts of the Optical Equipment of Microscopes. (After Zeiss.)

Note the positions and combinations of lenses which are used.

tance from the lens, in high-power close to it, in fact, in some, so close that the eyelashes rest upon the lens and sometimes appear in the field as dark bars."

2. The Objectives.—This part of the microscope contains the parts of the optical system that are nearest the object. Ordinary microscopes are fitted with three objectives which are carried in a revolving nosepiece. This nosepiece may be turned so that each objective as it comes under the draw-tube snaps into position. The objectives should be inserted and screwed into the socket of the nosepiece with considerable care.

Objectives are commonly designated as low-power dry, high-power dry and oil-immersion. These designations, however, are not the ones used by microscopists. Objectives are designated by the manufacturers by their equivalent focal length expressed either in inches or millimeters. An oil-immersion objective may be designated 1.8 mm., indicating that the object produces a real image of the same size as is produced by a simple converging lens the principal focal distance of which is 1.8 mm. The objectives may also be designated as $\frac{1}{12}$ inch, etc. Some manufacturers letter or number their objectives instead of designating them by

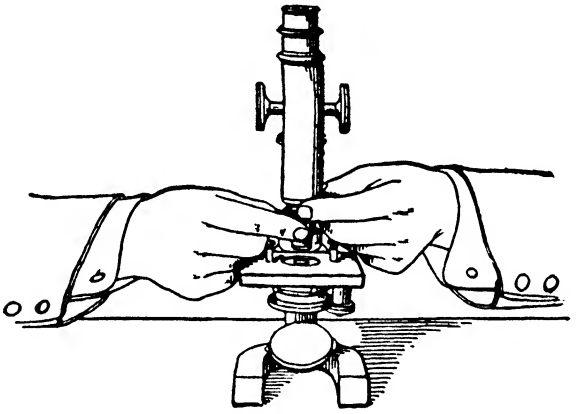


FIG. 22.—Proper Manner of Holding Objective when Attaching it to the Tube.

their equivalent focal lengths. The numbers in this case increase as the power of the objective increases.

All objectives are corrected to a certain tube length. These tube lengths are required in order that the objective be used with greatest definition. The tube length is regarded as the distance between the eye-lens in the eyepiece and the end of the tube into which the nosepiece is screwed. If the cover glass is thinner than that for which the objective is corrected the tube length must be made greater; if thicker, the tube must be shortened.

" **Cover Glass.**—It must not be forgotten that in microscopical work we are dealing with minute things, and this applies especially to the cover glass.

" In preliminary examinations of solid objects with low powers it may be dispensed with, but where fluids are used, whether with low, medium or high powers, it should always be used. A drop or small quantity of fluid placed upon a slide assumes a spherical form and, on viewing it with a low power, it

will be found to give a distorted field and cause disagreeable reflections and shadows.

" In medium and high powers, the front lenses will be so close to the water, urine, blood, etc., that capillary attraction will cause an adhesion to the front surface of the objective. By merely dropping a cover glass upon it these objections are overcome.

" Covers are commercially classified as No. 1, No. 2 and No. 3, but there is a variation within the limits of different numbers. The variation is about as follows:

No. 1, $\frac{1}{200}$ to $\frac{1}{150}$ inch, or 0.13 to 0.17 mm. thick
No. 2, $\frac{1}{150}$ to $\frac{1}{100}$ inch, or 0.17 to 0.25 mm. thick
No. 3, $\frac{1}{100}$ to $\frac{1}{50}$ inch, or 0.25 to 0.50 mm. thick

"Considered from the optical standpoint, No. 2 covers offer a range in thickness to meet the different standards as used by the makers of objectives. Test objects, which are prepared to test the resolving power of objectives and consist of diatoms, are generally covered with these thicknesses. The No. 1 are principally used with oil-immersion objectives as the working distance of these objectives is very short and more working distance is gained by using thin covers with them.

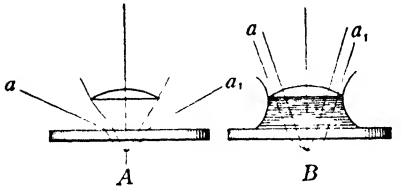


FIG. 23.—Showing the Effect of Immersion Oil in the Use of the Microscope.

A, Without oil between the slide and the lens in the objective, much light is lost by diffraction in the air. The rays *a* are bent to such an extent that they do not enter the draw tube. *B*, With immersion oil the diffraction caused by the air in *A* is prevented. The rays pass into the draw tube.

" The deviation from standard thickness affects the distinctness of the image according to its structure and in proportion to the increase in power. In the low powers there is no noticeable influence, but with the 4 mm. ($\frac{1}{6}$ inch) and 3 mm. ($\frac{1}{8}$ inch) it is so marked that with objects of fine structure a deviation of 0.05 mm. either thicker or thinner than the standard is sufficient totally to obliterate fine structure and have the outlines and coarse lines only apparent. Slighter variations affect the image proportionately.

" With oil-immersion objectives a variation in thickness is not appreciable, provided, however, that the fluid is of the proper consistency, as there is practically no refraction between the cover glass, immersion fluid and front lens of objective."

TABLE I

Name of Microscope	Tube Length	Thickness of Cover Glass
Bausch & Lomb Optical Co.	160 mm.	0.18 mm.
Spencer Lens Co.	160 mm.	0.18 mm.
E. Leitz.	170 mm.	0.17 mm.
Zeiss Optical Works.	160 mm.	0.16–0.17 mm.

"**To Attach Objective.**—Taking a low-power objective (16 mm.), remove from its box and see that its front lens is clean; elevate the tube of the stand by means of the coarse adjustment (*P H*) so that the nosepiece (*R N*) shall be at least 2 inches from the stage (*S*). (See Fig. 18.)

"To attach an objective properly is not always simple, *and cannot be done too carefully.* There is danger of dropping the objective on to the object, thereby damaging either or both, also of starting the threads wrong by holding the objective sideways, and thus injuring the threads.

"Grasp the upper knurled edge of the objective between thumb and forefinger of the left hand; bring the screw in contact with the screw of the nosepiece, and, keeping the objective in line with tube and gently pressing upward, revolve the objective with the thumb and forefinger of the right hand by the lower milled edge until shoulder sets against shoulder.

"**To Attach Objectives and Oval Revolving Nosepiece.**—Screw each objective into proper place in the nosepiece, with the 16 mm. ($\frac{2}{3}$ inch) opposite the opening through which the light passes.

"Hold the nose piece in the right hand, objectives down. Bring the revolving screw in contact with the screw in the tube, square with tube; with thumb and forefinger of left hand, turn milled edge of the revolving screw until it engages, swing nosepiece toward the front, and, holding it in this position, screw the ring home."

The Oil-immersion Objective.—Such objectives require the use of a drop of oil between the lens in the objective and the object. The oil serves to prevent a loss of light for it extinguishes two of the glass surfaces and thus reduces the possibility of refraction; the oil also permits the collection of the rays which would otherwise be lost. This is well shown in Fig. 23.

"**Oil-immersion Objectives.**—Immersion contact between the objective and cover glass is made with cedar oil. This oil is specially prepared so as to have the same refractive index as the front lens, and *great care should be used to keep it free from dust.* Apply the smallest quantity to the front lens, by allowing the superfluous amount to run from the rod, before objective is attached to nosepiece. If air bubbles are contained in the oil, remove it and apply a fresh quantity. This is exceedingly important, as the presence of dust or air bubbles in oil may destroy the definition of the best objective.

"Attach the objective and lower it until the fluid comes in contact with the cover; observe this by lowering the head to the level of the stage. Focus as with dry objectives.

"Special care must be observed if a low-power objective is used after an oil-immersion. The oil must invariably be removed from the top of the cover glass by wiping with lens paper. The front of the objective should always be cleaned in the same manner immediately after it has been used. Oil allowed to dry through oversight may be removed by using lens paper moistened with Xylol.

"**Care of Objectives and Eyepieces.**—Every outfit should be provided with a camel's hair brush and a well-washed piece of linen. On account of its

fine texture, chamois skin is desirable, but only after it has been repeatedly washed. No dust should be permitted to settle upon a lens nor should the finer one come in contact with any of the surfaces. Occasional cleaning is desirable even when the objectives and eyepieces are not used, as dust settles upon the outer as well as the inner surfaces of the eyepiece and the rear surface of the objective, and creates a cloudiness in the image.

"When not in use objectives and eyepieces should be kept in their receptacles. If objectives are left attached to the microscope either singly or on revolving nosepieces, leave the eyepiece in the tube so that no dust can enter and settle upon the rear lens of the objective.

"Objectives especially should be kept where they are not subject to extreme and sudden changes of temperature as the expansion and contraction may cause the cement between the lenses to crack.

"*Eyepiece.*—Visible defects in the field are always traceable to impurities in the eyepiece, not in the objective, and are easily recognized by revolving it. Indistinctness in the image or loss of light may be due to soiled or coated surfaces in either eyepiece or objective.

"Dust, if on either the eye-lens or the field-lens, is apparent as dark, indistinct spots.

"To clean the surfaces, breathe upon them and, giving a revolving motion to the eyepiece, wipe with well washed linen and finally blow upon the surface, or use camel's hair brush to remove particles of lint.

"At regular periods unscrew the eye-lens and field-lens and clean the inner surfaces.

Objective.—This should be used with the utmost care. The systems should never be separated, even if they can be unscrewed, as they are liable to become decentered and dust may enter.

"Avoid all violent contact of the front lens with the cover glass. The oil-immersion objectives particularly require the best care.

"Occasionally examine the rear surface of the objective with magnifier, and if dust be present remove with camel's hair brush.

"Clean an immersion objective immediately after it has been used by removing the fluid with lens paper.

"While cleaning give the objective a revolving motion.

"If the immersion oil should have become thick, or any substance adheres to the surface, it may be removed by using lens paper moistened with Xylol."

3. The Condenser.

"**Purpose of the Condenser.**—The purpose of the condenser is to give an amply illuminated field when the illumination is otherwise insufficient.

"The condenser mounts fit into the substage from below and are provided with an iris diaphragm, which controls the amount of light entering the condenser and the angle of the emitted cone. They are also provided with a swing-out carrier for holding a blue glass disc or a dark ground stop.

"Use only the plane mirror with the condenser. A condenser is so constructed that parallel rays of light are brought to a focus above the upper surface of its uppermost lens and in the plane of the object. If the concave

mirror is used the convergence of light is more rapid and the apex of the cone of light is within the condenser and its effectiveness depreciated.

"Centering the condenser is the act of bringing its optical axis coincident with the optical axis of objective.

"To verify correct centering, two easy methods may be followed:

I. Use a 48 mm. (2 inch) objective and focus through the condenser on to the diaphram, which is reduced to its smallest opening.

II. Use a 16 mm. (2 inch) objective: focus upon upper surface of condenser or upon an object, which should then be removed; elevate the objective with coarse adjustment until a dimly defined dark spot appears in the field and with proper focusing is about one-third of the diameter of the field.

"Centering of the condenser does not imply that the cone of illumination is also centered, and it is fully as important to secure the correct conditions in one as it is the other.

"**Centering of Illumination.**—The mirror may be so adjusted that the light will be directed toward the periphery of the condenser, and when lamplight is used the light may be so placed as to give all gradations of oblique illumination from the central to the limit of aperture, although the condenser may be centered.

"With daylight have evenly illuminated field.

"With lamplight attach 16 mm. ($\frac{2}{3}$ inch) objective; open diaphragm to full extent and focus upon the minute image of flame; adjust mirror so that the image will be in the center of the field.

"**To Focus Condenser.**—In all the various forms of mountings the condenser is so mounted that at the uppermost limit of adjustment its upper surface is slightly below the surface of the stage so that it is almost in contact with the slide.

"With all objectives having a numerical aperture less than 1.0 the condenser may be used dry, i.e., without oil.

"In the use of the condenser with oil-immersion objectives the custom prevails of using the condenser dry. It is well to point out, however, that both the condenser and the objective lose in their efficiency when the former is used dry, and for critical work the condenser should be in immersion contact with the slide.

"To make immersion contact between condenser and slide place a drop of oil on the top of condenser, drop the slide upon the stage, first turning the clips to one side.

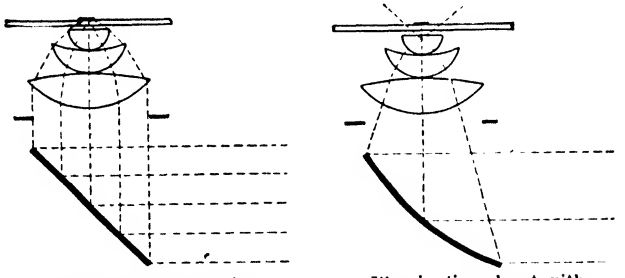
"With immersion objectives the proper focusing of the condenser becomes a matter of nice distinction to obtain best results and can only be reliably accomplished by considerable practice and experience. To obtain best position:

"Use a 16 mm. ($\frac{2}{3}$ inch) objective; focus upon the object; adjust condenser until image of window-sash or flame is in the same plane with object.

"*Relation of Aperture of Condenser to Objective.*—In the study of bacteria and other microorganisms, the objectives used being of wide aperture, it is sought to have them stand out boldly in a bright field. This is accomplished by bringing the diaphragm to its full aperture. On all other objects, however, too much illumination decidedly injures definition by obliterating detail.

"Little experience is required to judge when the condenser has its proper opening. When correct, the image will stand out sharply defined without any appearance of fogginess, and as the diaphragm aperture is reduced it will be noticeable by the decrease in the amount of light. By removing the eyepiece and looking at the back of the objective the relative aperture of the condenser to that of the objective may be easily seen, as the outlines of the diaphragm are sharply defined. In testing for this, start with the smallest aperture of the diaphragm and gradually increase its diameter. If the opening in the diaphragm appears to have the same opening as the back of the objective, the condenser has the same angular aperture. In the following instructions for the proper use of light from the condenser, the size of opening of its diaphragm as it appears by viewing the back of the objective is called apparent aperture. By experience the following conditions have been found to give most satisfactory results:

"In oil-immersion objectives on bacteria use the full opening of diaphragm.



Illuminating object with condenser and plane mirror The right way.

Illuminating object with condenser and concave mirror. The wrong way.

FIG. 24.—Showing the Effect of Plane and Concave Mirrors and the Lenses in Condensers.

"On diatoms reduce the apparent aperture to about two-thirds opening in objective.

"In histological and other dense objects use the apparent aperture equal to about one-half the opening of back lens in objective.

"In dry objectives the aperture of the condenser should always be less than that of the objective.

"*Oblique Light with Condenser.*—Oblique light may be obtained by setting the mirror alone in such a position that the light reflected from it shall enter the condenser only at one side, leaving the balance of it unused. This, however, is only advisable when the condenser mounting has no other provision for obtaining oblique light. In the mountings having such provision oblique illumination may be obtained by two methods:

"I. Focus objective; reduce the apparent aperture to that of the rear lens of the objective, turn the plate carrying the lateral adjustment around so that the pinion button is at the side. Work the pinion button so that the opening will move from the center to the periphery of the condenser.

" II. Proceed as above with this difference: Remove eyepiece and view the bright circle of light as it passes from the center to the periphery of the rear lens.

" When the circle of light has passed beyond the limit of aperture of the objective the field will become dark.

" In objects with striated structure, the illuminating rays should be brought to a position at right angles to the striae, either by rotating the object to the proper position, or by turning the diaphragm plate."

4. The Mirror.—The mirror usually has two reflecting surfaces, one plain or flat, and the other concave. The plain mirror reflects the light rays parallel to one another while the concave mirror concentrates the light.

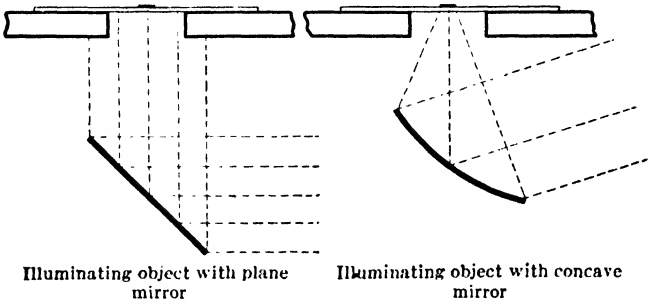


FIG. 25.—Showing the Effect of Using the Concave and Plane Mirror alone without Substage Condenser.

USE OF THE MICROSCOPE

The Spencer Lens Company has published the following advice about what not to do with the microscope.

Don't allow dust and dirt to settle on the microscope.

Don't carry the microscope by the arm unless the fine adjustment is protected.

Don't use alcohol on the microscope.

Don't expect too great a range in the fine adjustment.

Don't take the fine adjustment apart.

Don't bring the objective into contact with the cover glass.

Don't fail to focus up before turning the nosepiece unless you *know* the objectives are par-focal.

Don't forget that high powers have short working distances

Don't focus down with the eye at the eyepiece.

Don't fail to secure good, even illumination.

Don't drop the objectives and oculars.

Don't try to take an objective apart.

Don't try to work with dirty lenses.

Don't try to clean the lenses with a dirty cloth.

Don't fail to clean oil from an immersion lens immediately after using.

Don't try to work with an immersion lens when there are air bubbles in the oil.

Don't use high powers when low ones will do.

Don't use higher oculars than necessary.

Don't expect a lens to work at its best unless used on a cover thickness, and with a tube length, for which it is corrected.

Don't shut one eye.

Don't get discouraged if desired results do not come immediately.

"**Finding an Object.**—The slide on which the object is mounted is placed upon the stage under the spring clips or in the mechanical stage to a point where the object comes as nearly as possible over the center of the opening in the stage.

"With low-power objectives (used on coarse and large objects) it may be found, after focusing, that only a portion of the object will show itself in the field, and upon moving the slide it can readily be brought to the center. In this connection it will be noted that a movement of the object to the left is an apparent movement to the right in the field. This is because the image in the eyepieces is reversed in position from that of the object. In case of a small object not found after the objective is known to be in focus (as may be told if the mounting medium or small particles of dust on the cover glass are visible) move the slide about on the stage by grasping one end with the thumb and forefinger, when the object can usually be recognized by its shadowy outlines as it flits across the field. The difficulty of finding an object or a particular spot in it becomes greater as the power increases and even in experienced hands sometimes becomes quite vexatious. Recourse may be had to two methods:

By using a lower-power eyepiece.

By using a low-power objective as a finder.

"A larger field is thus obtained in which the object may be more easily found, and then moved to the center of the field. The low-power objective is then removed and the high power attached; or in case the revolving nosepiece is used, after using the low-power objective as a finder, turn the high-power objective into position, care being taken not to touch the slide, and focus in the manner to be described. If object is then not in the field, it will certainly be very close and ought to be easily found.

"*To Illuminate the Object.*—This is an extremely important detail, and should always be carefully done, as one may easily fail to obtain the best results, may be led to wrong conclusions, or may injure the eyes. The mirrors of the microscope are usually plane and concave, and are adjustable so as to be able to reflect the light from any source in front or at the side of the microscope.

"The plane mirror reflects the light in its initial intensity and is used with low-power objectives. The concave mirror concentrates the rays on the object, thereby giving intensified illumination, and is used with medium and high-power objectives. When a substage condenser is used, the plane mirror is employed.

" The sources of light are either daylight or artificial light; if the former, the light of a northern sky is preferred, and if the latter a flat-wick oil lamp, Welsbach gas burner or electric lamp. An ordinary gas flame should not be used on account of the difficulty of obtaining an even illumination and the constant flickering which is injurious to the eye. When using a flat-wick lamp the narrow edge of the flame should be used, as this is more intense than the broad side.

" When using daylight place the microscope, as nearly as possible, directly before a window, and when a lamp is employed, have it on the table either in front or at the right side of the microscope and within easy reach. Light is either transmitted or reflected. When the former, it is to illuminate transparent objects and passes through the object from below the stage into the objective. With opaque objects this is impossible and reflected light is required, when it is directed on to the object from above and illuminates its upper surface. In the following instructions it is assumed that transmitted light is used unless otherwise stated.

" Before lighting an object make certain that the mirror bar is in exactly central position, and set the mirror at such an angle to the light that it will be reflected upon the object. This can be done more quickly at the outset by observing the object or the opening of the stage, keeping the head at one side of the tube. Now remove the eyepiece, and observe the light through the objective. It should be central and of equal intensity, which, with daylight, is sometimes difficult to obtain as the sash of the window may be reflected and show itself in the field as dark bands, or in the case of lamplight the blue portion of the flame may appear as a dark spot. These are only preliminary directions but will suffice for a beginning. There will be little difficulty in obtaining proper illumination at the outset if one will observe the following:

" Remove the eyepiece and, looking through the back of the objective, have

Central illumination.
Even illumination over entire field.
Mellow illumination.

" Defects in illumination which may not be apparent will show when the eyepiece is replaced, and are indicated,

When dark points or shadows appear in the field.
When the outlines of an object are bright on one side and dark on the other.
When the object appears to lie in a glare of light.

" In the first two cases the correction can be made by suitably adjusting the position of the mirror, in the last by reducing the amount of light by the use of the iris diaphragm between mirror and object.

" It is now generally conceded that observations with the microscope may be made to any extent without any detrimental results to the eyes, provided, however, that the conditions of light are just right. It is a good rule to follow, to use as small an amount of illumination as will comfortably show the struc-

ture which is being studied, and it may also be safely accepted that, if the eye tires or feels uncomfortable, the light should be moderated.

" Illumination is either

Central or axial, when the center of the mirror is in the optical axis, or
Oblique, when the mirror is swung to one side, which in objectives of wide aperture will disclose structure that cannot be seen with central illumination.

" To focus an objective is to adjust its relation to the object so that a clear image is obtained. Focusing should involve no danger to the front lens of the objective, or to the cover glass, by their coming in violent contact. With the lower-power objectives, in which the working distance is great, there should be little danger; with the higher-power objectives, in which the working distance is so small that the front of the objective is very close to the cover glass, there is considerable.

" To focus low-power objective:

"Attach objective to the nosepiece. Lower the head to the level of the stage, to be able to see the front of the objective; lower the tube by the coarse adjustment until the front of the objective is within ¼ inch of the object; look through the eyepiece and slowly elevate by the coarse adjustment until the image is distinct. Use fine adjustment.

" The upward movement should be slow so that, if the object be faint, it is not missed and the adjustment not run beyond its focal distance; or it is possible that, in the case of a very minute object, it may be out of the center, and thus out of the field of vision, in which case the surface of the cover glass, or the minute particles of dust upon it should be distinguishable.

" The object will first appear with faint outlines and indistinct; then gradually more distinct, and finally sharply defined, and if adjustment goes beyond this point it will gradually become more dim, in which case return to the point of greatest distinctness.

" To focus high-power objective:

"Attach objective to nosepiece. Lower the head to the level of the stage and look between objective and cover glass toward a window or a flame. Slowly lower the objective with the coarse adjustment until the front of the objective is nearly in contact with the cover glass; look into the eyepiece, slowly elevate the tube by the coarse adjustment until the image appears. Use fine adjustment.

" It is also advisable while watching for the image to appear to move the object slowly in different directions, as the flitting of shadows of colors across the field will give indication that the objective is nearing the focal point.

"Always focus upward. In case a low power is exchanged for a higher objective, or when the low power has been used as a searcher, i.e., to find a certain object in a collection, or a certain locality in a specimen, the tube should first be elevated, as working distance in the high power is too short to admit of screwing it into the nosepiece; then detach the low power, attach the high power and proceed to focus in the order given.

" Objectives which make up the regular outfits are so adjusted as to be par-focal, i.e., are so fitted to the nosepiece that as either one is swung into

position, it is so nearly focused as to require only the use of the fine adjustment. Very low-power objectives have so much longer working distances than hose which make up the regular outfit that it is generally impossible to make them par-focal. The 16 mm. ($\frac{2}{3}$ inch) and 4 mm. ($\frac{1}{6}$ inch) or 3 mm. ($\frac{1}{8}$ inch) on the double nosepiece, and 16 mm., 4 mm. or 3 mm. and the 1.9 mm. ($\frac{1}{12}$ inch) oil-immersion on the triple nosepiece are so made. But in a combination of a lower power with 16 mm. and 4 mm. on the triple nosepiece, it is impossible to get the lower power par-focal with the others.

"It is highly important to preserve proper tube length. The objectives are adjusted for exactly 160.0 mm. distance or tube length, measured from the upper rim or draw-tube to the lower edge of nosepiece against which the shoulder of objective screws."

EXERCISE NO. 1: THE MICROSCOPE

1. Take the microscope from its case and place it on the desk.

2. Pull out the draw-tube to the required length. (Why?)

3. Remove the eyepiece and, by looking into the draw-tube, arrange the illumination. The field should be free from any obstructions, window bars, etc., that may be imaged by the mirror.

4. Replace the eyepiece and see that the low-power objective is in place.

5. Prepare a "mount" of starch as follows: Scrape the surface of a peeled potato and drop the material taken upon a slide, covering with a cover glass. Do not use too much of the potato under the cover glass. If desired the dry potato starch may be used. If time permits, wheat starch may also be used. With these materials, place a drop of distilled water on the slide, sift a little dry starch into it, cover with a cover glass and examine.

6. Place this "mount" on the stage of the microscope and, by means of the coarse adjustment, focus the body tube downward until the objective nearly touches the cover glass, being careful not to touch it. Then, with the eye at the eyepiece, focus upward with the coarse adjustment until the specimen comes into view. After this use the fine adjustment to bring out the details.

7. Examine this slide with (*a*) the low-power objective, (*b*) the high-power dry lens and (*c*) the oil-immersion objective. Make drawings in each case and draw in such a manner that the relative magnification is shown. Indicate under each drawing the number of diameters of magnification obtained with each objective, as given on the chart in the laboratory.

8. Repeat 5, 6, and 7, using some other substance, such as epithelial cells secured by scraping the back of the hand, wing of a fly, thin onion skin, hair, etc. Make drawings and label carefully.

9. While examining the above mounts, vary the opening of the iris diaphragm. This may be done by removing the mount from the stage, and the eyepiece from the tube.

Record the data from this experiment on the special sheet provided, and record under each the magnification.

10. On another sheet of paper [2] record the following observations:

a. The name of the manufacturer of the instrument.

b. The number of the microscope and the numbers of the various parts.

1. Oculars (usually one).
2. Objectives (usually three).

c. Prepare a table showing the magnification possible with the instrument with which you will work.

d. In what direction should the fine and coarse adjustments be turned to lower or raise the tube? (Clockwise or counter-clockwise?)

MICROMETRY: MEASUREMENT OF MICROORGANISMS

The microscopist frequently wishes to measure the size of the objects which he examines or to know the diameters of micro-

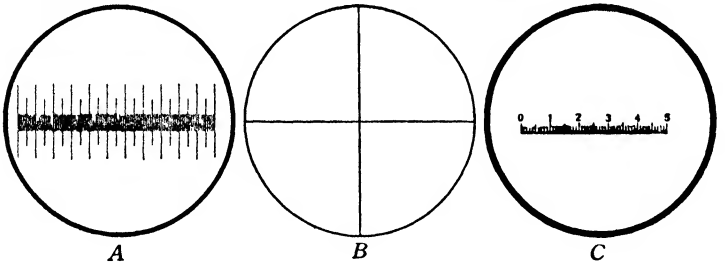


FIG. 26.—Showing Micrometers Necessary for the Measurement of Microorganisms. (After Zeiss.)

A, Stage Micrometer mounted on an Object Slide. *B*, Eyepiece with Cross Lines for Centering Revolving Stages. *C*, Ocular Micrometer to be fitted on the Field Stop of the Ocular. (About 1½ Times Actual Size.)

scopic fields. The apparatus used in such work is called a micrometer. There are several types of micrometers, of which only the simplest need be discussed here. The microscopist makes his measurements with a micrometer which fits into the ocular;

[2] One sheet may be used for recording all of the observations in this experiment. Special printed sheets may be distributed by the instructor.

TABLE II

Spencer Lens Company Magnification Table

Tube Length, 160 mm. Image Distance, 250 mm.

Objectives, Mm.	Initial Magnification	Oculars								Objectives, Mm.
		4x	5x	6x	8x	10x	12x	15x	20x	
48	2 2	8	11	13	18	22	27	33	44	48
40	2.8	11	14	17	22	28	33	42	56	40
32	4	16	20	24	32	40	48	60	80	32
30–22	2–4.5	4–9	5–12	8–19	10–24	15–35	18–43	20–48	30–70	30–22
25.4	6	24	30	36	48	60	72	90	120	25.4
16	10	40	50	60	80	100	120	150	200	16
12	15	60	75	90	120	150	180	225	300	12
8	20	80	100	120	160	200	240	300	400	8
5	36	144	180	216	288	360	432	540	720	5
4	44	176	220	264	352	440	528	660	880	4
3	60	240	300	360	480	600	720	900	1200	3
1 8	95	380	475	570	760	950	1140	1425	1900	1.8
1 5	109	436	545	654	872	1090	1308	1635	2180	1 5

TABLE III

Magnification Table

(Bausch & Lomb Optical Co., Rochester, N. Y.)

Tube Length 160 mm. Image Distance 250 mm.

Objectives, Mm.	Eyepieces					Objectives, Inch	Working Distance, Mm.
	5x	6.4x	7.5x	10x	12.5x		
48.0	10	13	15	20	25	2	53
32.0	20	26	30	40	50	$1\frac{1}{3}$	38
16.0	50	64	75	100	125	$\frac{2}{3}$	7.0
8.0	100	130	150	200	260	$\frac{1}{3}$	1.6
4.0	215	275	320	430	560	$\frac{1}{6}$	0 6
4.0_L	215	275	320	430	560	$\frac{1}{6}$	0.3
3.0_S	285	365	420	570	740	$\frac{1}{8}$	0.2
1.90	475	610	720	950	1260	$\frac{1}{12}$	0.15

TABLE IV

Table of Magnifications of Achromatic Objectives with Huygenian Oculars

(Calculated for a Tube Length of 160 mm. and an Image Distance of 250 mm.)

Objective... ..		a_0	a_1	a_2	a_3	a^*	aa	A	AA	B	C	D, DD	E	F	Plankton-Searcher	D^*	J	$\frac{1}{12}''$
With Ocular	1	4.5	7	11	20	3– 8	24	42	39	58	100	175	275	420	26	175	420	410
	2	7	10	15	26	5–12	31	54	50	74	125	225	345	530	33	220	530	515
	3	11	16	23	38	8–18	46	79	73	110	180	325	505	770	48	315	770	750
	4	14	20	28	47	10–22	57	97	90	130	225	405	620	940	60	390	945	920
	5	23	31	43	68	16–33	83	142	131	190	325	580	900	1350	87	560	1360	1340

The tube length is reckoned from the shoulder of the objective screw to the upper end of the draw-tube on which the ocular rests. In using nosepiece or objective-changer the length of the nosepiece (15 mm.) and that of the objective-changer (22 mm.) should be taken into account, and the draw-tube shortened by the like amount, so that the distance between objective shoulder and ocular amounts to 160 mm. It is essential that the correct tube length be maintained in all objectives of large aperture (0.85 and up), as otherwise a considerable impairment of the image can take place.

For such objectives with fixed mounts, excepting those for homogeneous immersion, a mean *cover-glass thickness* of 0.17–0.18 mm. should also be adhered to. In objectives with correction adjustment the thickness of the cover glass used for the specimen must be determined and the correction collar adjusted accordingly.

Homogeneous immersion objectives are, within wide limits, independent of the thicknesses of cover glasses. Thickened cedarwood oil ($n_D = 1.515$) should only be employed as *immersion fluid* for homogeneous objectives. Each time after use the oil should be carefully wiped off with a clean, soft piece of linen dipped in benzine.

it is, consequently, known as an *ocular micrometer.* The ocular micrometer is dropped on to the diaphragm of the ocular after the eye-lens and its fitting have been unscrewed. After the ocular micrometer has been placed, it must be standardized by means of a stage micrometer. Such standardization is necessary because the magnifying power of a microscope is dependent upon several different factors, such as the draw-tube length and the magnifying power of the oculars and objectives.

The ocular micrometer is standardized with a stage micrometer. The stage micrometers usually have a ruled line of 5 mm. divided into tenths. The object of standardization is to evaluate each division on the ocular micrometer in terms of the divisions on the stage micrometer, which is the standard.

When the value of each division on the ocular micrometer has been determined, the stage micrometer may be removed, and the glass slide with the object to be measured may be substituted. By making the lines on the ocular micrometer coincide with the edges of the object, whether it be a diatom, bacterial cell, mold spore, yeast cell, etc., the size of the object may be determined by multiplying the number of divisions covered by the object by the factor arrived at during the standardization of the ocular micrometer with the stage micrometer.

CHAPTER III

CULTURE MEDIA

THE microorganisms studied in the laboratory must, of course, be maintained in an artificial or controlled environment after they have been removed from their natural habitat. This artificial environment should be as much like the natural one as possible, and should be kept as constant as possible. In order to accomplish this the microbiologist has adopted special apparatus, which has been described in the first chapter, and special food materials, which are called *media*[1] (singular, *medium*). These media contain most of the materials demanded by the organisms under examination and, as far as possible, are devised and adapted to suit the characteristics of the organism. For instance, some bacteria are unable to tolerate the presence of organic matter; hence, they are cultivated in media made up of pure inorganic salts entirely. Other bacteria require the presence of haemoglobin, and media containing this substance are accordingly used.

Standard Methods.—In order to have uniformity it was necessary that efforts be made to standardize the procedures and technic of bacteriology. For instance, if no attention were given to formulæ for the preparation of media, bacteriologists the country over could not use the same media, and uniformity, in this respect, would be impossible. Data collected with such media in different laboratories could not be compared. Thus, there could be no standard methods of procedure and no standards of quality for food materials, etc.

The first attempt at standard methods and media was probably made by the American Public Health Association. The early efforts of this organization finally culminated in the preparation of a report entitled "Standard Methods for the Examination of Water and Sewage." Later, another manual for milk was pub-

[1] Some writers use the term *medium* for the singular and the term *mediums* for the plural.

lished under the title " Standard Methods for the Examination of Milk." The printed proceedings of this organization have always contained papers and reports on technic. In this manner there are available to-day standard methods for the examination of most foods and specimens from diseased conditions.

The Society of American Bacteriologists has also had a Committee on Bacteriological Technic which has been attempting to standardize technic. The progress which they have made is embodied in a pamphlet entitled " Manual of Methods for Pure Culture Study of Bacteria." [2]

Characteristics of a Good Medium.—A good medium, of course, is one that simulates as closely as possible the natural conditions under which the organism grows, or one that has been found to permit luxuriant growth under artificial conditions. The selection of a medium is often a difficult problem, especially for microorganisms that are fastidious in their food requirements. The following characteristics and conditions are considered in the preparation and use of various media.

1. Must contain the proper amount of moisture. A good medium probably approaches an isotonic solution.
2. Must be sterile.
3. Must contain the necessary kinds and amounts of food substances.
4. Must possess the proper reaction.
5. Must approach isotonicity - must have the same density as the bacterial cell.
6. Must frequently have special substances demanded by certain microorganisms.

CONSTITUENTS OF MEDIA

The following materials are used in most common media. In contrast with mixtures of these materials, which are used in different combinations, are the natural media, such as milk, potato, carrot, etc.

Water.—If possible, distilled water should be used for the preparation of media. No particular error is introduced, perhaps, if tap water is used, but it will be obvious that uniform media may not be prepared. The chemical constituents of water vary

[2] Copies of this pamphlet may be secured from the chairman of the committee, Dr. H. J. Conn, Agricultural Experiment Station, Geneva, N. Y.

greatly even though they may be present in very small amounts. Bacteria require very small amounts of food and may find certain tap waters in themselves satisfactory media. A more uniform medium is secured with distilled water.

Agar.—Field (Chemical Age, December, 1921) has described the preparation of agar-agar (not the medium) as follows:

"Agar-agar is the commercial convenient name applied to the dried, gelatinous extract of certain species of red algae. The algae most commonly used are *Gelidium corneum*, *G. cartilagineum*, *Gracilaria confervoides*, *Eucheuna spinosum*, and some species belonging to the genera *Glocopeltis* and *Gigartina*. Of these, *Gelidium* varieties produce the best quality of agar. Most of the agar of commerce is produced at present in Japan, China, Malay, and Ceylon, although there seems to be no reason why the United States should not manufacture more than enough for its needs. During the year 1920, this country imported 240 tons, having a value of nearly a half million dollars.

" Preparation: The preparation of agar (not the medium) involves quite a number of steps, but on the whole is a simple process:

" (1) The seaweeds are collected by hand or rakes and spread upon the beach to dry and bleach in the sun.

" (2) The dry weed is beaten or pounded by hand or passed through a concrete mortar-and-pestle battery to free it from clinging shells, incrusting Bryozoa, sand, and other foreign matter, and is then alternately washed and sun-dried again until thoroughly bleached and cleaned. This treatment requires from two to several days. Some manufacturers are said to shorten the process by bleaching with chemicals instead of sunlight.

" (3) The bleached raw material is boiled with about 50 times its weight of water in an iron kettle for from three to five hours to extract the gelose in soluble form. The solution is then separated from the insoluble matter by filtering the mass first through coarse cloths and then squeezing it through linen bags in a press.

" (4) The filtered jelly is next poured into wooden trays over 2 feet long, 1 foot wide, and 3 inches deep, to cool. As the filtrate cools it solidifies into a hard jelly which the Japanese call " tokoroten." In this form it is cut by means of sharp knives into blocks 1 foot long and 2 inches square. These blocks are in turn pressed through a coarse wire grating which cuts them into bundles of slender straws.

" (5) In this condition the ' tokoroten ' is subjected to a freezing temperature of $-5°$ to $-15°$ C., either out of doors or in an artificial freezer, until the sticks are frozen solid. This causes the water to crystallize out, and when it is melted the substances soluble in cold water drain off in solution, leaving the gelose in pure condition. By repeating the freezing and thawing process and at the same time drying the material in the sun and open air, a pure agar which is insoluble in cold water is prepared.

" (6) Before the sticks are entirely dry they are sometimes put through a forcing machine which flattens each fine strip into a transparent sheet. They are then dried in the sun and tied in bundles weighing from ½ to 3 pounds each.

"Agar-agar is prepared also in the form of sheets 8 to 12 inches long and

1 to 1½ inches wide, and as rectangular blocks about 8 inches long and 1 inch square.

"The chemical composition of agar-agar has been most carefully studied by Carl R. Fellers.[3]

Analyses of 15 samples collected from various sources and representing different brands gave the following results:

	Percentages
Moisture	15.75–17.84
Protein ($N \times 6.25$)	1 63– 2 94
Nitrogen-free extract	72.72–78 21
Ether extract	0.17– 0.45
Crude fiber	0.39– 1.60
Ash	3.08– 5.68
Silicon dioxide	0 31– 1.11

Agar is a complex carbohydrate classified under the poly-saccharides. The following classification of the carbohydrates will show the position of agar-agar in the classification of sugars and will be useful in following the changes produced by micro-organisms.

I. Mono-saccharides
  1. Pentoses
    a. Arabinose
    b. Xylose
    c. Rhamnose
  2. Hexoses
    a. Glucose
    b. Fructose
    c. Galactose

II. Di-saccharides
  1. Maltose
  2. Lactose
  3. Iso-maltose
  4. Sucrose

III. Tri-saccharides
  1. Raffinose

IV. Poly-saccharides
  1. Gums
  2. Starches
  3. Cellulose
    a. Cellulose
    b. Hemicellulose
      (1) Pentosans
      (2) Hexosans
        (*a*) Galactans
          1. Agar-agar

[3] Jour. Ind. Eng. Chem. Vol. 8, No. 12, pp. 1128–1133.

Agar is quite hygroscopic. The Digestive Ferments Co., by an experiment extending over twenty months, showed that agar exposed to the atmosphere under ordinary conditions of the laboratory stabilizes its moisture content around 12 to 15 per cent. Samples of agar with an initial water content ranging from 1 to 20 per cent reached a constant content of 13 per cent in a short time. Such data show that the moisture content of agar varies.

Gelatin.—Gelatin is a protein prepared by the acid hydrolysis of collagen. Collagen is, in turn, secured from the cartilage, connective tissue of animals, etc. Since gelatin is a protein it may be decomposed by bacteria secreting the necessary enzymes (proteases) and used as food. Unlike agar medium, gelatin medium liquefies (turns from a jelly mass to a thin liquid).

Dakin has reported the following amino acids in gelatin.[4]

	Per Cent		Per Cent
Glycin	25.5	Tyrosin	0.01
Alanin	8.7	Prolin	9.5
Aminobutyric acid	none	Hydroxyprolin	14.1
Valin	none	Aspartic acid	3.4
Isoleucine	none	Glutamic acid	5.8
Serine	0.4	Histidin	0.9
Leucine	7.1	Arginin	8.2
Phenylalanine	1.4	Lysin	5.9
		Ammonia	0.4

Gelatin is easily spoiled by too much heat. When incorporated into plain broth to make gelatin medium, it must be sterilized with some care, else a product will result which will not solidify.

Peptones.—Peptones are cleavage products of protein. Bacteria must have nitrogen in media in a readily available form. This element cannot be taken from all nitrogenous compounds. The nitrogen in proteins, for instance, cannot be utilized unless it has been simplified by cleavage processes. The work of Bainbridge in England and of Rettger and his pupils in America has revealed that bacteria, even those species which are strongly proteolytic, are unable to attack pure native proteins. Rettger showed that even a simple substance, such as coagulated egg allbumin, was quite valueless as food for bacteria unless simpler substances were supplied for the initial energy and growth.

[4] Dakin, H. D., Amino Acids in Gelatin. Jour. Biol. Chem., 44 (1920), 499–529.

After growth has started and enzymes have been elaborated, the protein molecules are susceptible to decomposition.

Chemically speaking, the peptones are secondary cleavage products of proteins. They are defined as hydrolytic decomposition products of proteins; soluble in water, not coagulable by heat; not precipitated by saturation with ammonium sulfate. They are generally diffusible and give the Biuret reaction. (Mathews' "Physiological Chemistry.")

The commercial peptones, however, are quite different from the peptones of the chemist who is using the term in its narrow chemical sense. It has been found by Rettger and his colleagues that even the true peptones are quite unsuited for bacterial metabolism and that it is the content of amino acids which makes the commercial peptones useful. Bacteria are able to utilize amino acids. These units of the protein molecule are able to pass through the cell wall of the bacteria and be decomposed within the cell for building and energy. In this manner bacterial metabolism is similar to human metabolism.

Use of Indicators in Media.—Very useful media have been developed by adding certain indicators directly to the medium before inoculation. These media are used for detecting the presence of acid formation by the microorganisms which grow therein. One of the oldest is litmus milk. Later other indicators have been used, such as fuchsins, brom-cresol purple, etc.

Beef Extract and Beef Infusion Media.—Meat extract is also added to bacteriological culture media in order to supply certain substances which stimulate bacterial activity. Meat extract is not a food itself, since its method of preparation removes almost all food constituents. It does contain enzyme-exciters, such as the phosphates, which cause accelerated growth of the bacteria. It is prepared by soaking lean beef in water, filtering and concentrating the filtrate. The resulting mass is dark-colored, thick and pasty.

In place of beef extract some bacteriologists use beef infusion or beef juice. This is prepared by soaking 500 grams of lean beef in distilled water over night and filtering until a clear filtrate is obtained. This filtrate may be substituted for the beef extract and water in the following formulæ for the preparation of media. Meat infusion may contain more of the natural accessory substances (vitamines?) than the commercial meat extract; however,

the need of accessary substances by bacteria is not yet an extirely settled question.

CLASSIFICATION OF BACTERIOLOGICAL MEDIA

I. Liquid Media
   A. Natural liquid media
      1. Milk, litmus milk
      2. Fruit juices: apple, grape, prune, etc.
      3.
   B. Artificial liquid media
      1. Plain broth or bouillon, carbohydrate broths, etc.
      2. Synthetic media (from pure salts)

II. Liquefiable Solid Media
   A. Agar media
      1. Plain agar, carbohydrate agars, etc.
      2.
   B. Gelatin
      1. Plain gelatin, carbohydrate gelatin
      2.
   C. Combinations of Agar and Gelatin

III. Solid Media
   A. Natural
      1. Potato, carrot, etc.
   B. Artificial
      1. Silica jelly

LIQUID MEDIA

Liquid media were used at first in bacteriology. They were made of various extracts and permitted the garnering of much information about bacteria. Significant advances came more rapidly after the introduction of solid media or media that could be liquefied and later solidified in Petri dishes.

Beef Extract Broth.—This is the basic medium for many media. It is composed of

Beef extract	3 grams
Peptone	5 grams
Water	1000 c.c.

Earlier investigators added salt to this medium. It is not used to-day. The formula given above contains the ingredients which are used in most of the common media. Other materials may be added to yield various modifications.

Procedure:

1. Carefully clean the media pot or other dish and put therein 1 liter of distilled water.[5]

2. Add 5 grams of peptone and 3 grams of meat extract. The latter is a pasty substance and may be weighed out on paper. When the paper is placed in the water, the meat extract usually slips off, after which the paper may be removed.

3. Boil until the ingredients are *thoroughly dissolved.*

4. Adjust the reaction to neutrality by means of sodium hydroxide with brom-thymol blue as the indicator. This may be done by putting drops of the indicator in the depressions of a test tablet.[6] Drops of the broth may then be transferred to these depressions in order to see if the desired reaction has been reached. Add the sodium hydroxide very slowly to the broth, testing the reaction frequently until a grass-green color is obtained. If the initial reaction is alkaline, neutralize with hydrochloric acid.

5. Filter through paper, several times if necessary, and put the clear filtrate into test tubes or flasks, according to the directions of the laboratory instructor.

6. Sterilize in the autoclav at 15 pounds for twenty-five minutes.

This beef-extract broth medium is the base for many other media. For instance, as is shown in the following paragraphs it is the base for nutrient agar and nutrient gelatin. Under some circumstances agar and gelatin may be prepared from beef-extract broth if it is available.

Beef-infusion Broth.—As stated above, beef infusion may be substituted for beef extract. This is prepared by soaking 500 grams of lean chopped beef in 500 c.c. of distilled water. The meat should be placed in the refrigerator over night; in the morning it may be strained through cloth. A hand press is desirable for securing all of the liquor from the meat. This filtered extract contains the " extractives " from the meat. This material may be used as the base for the preparation of agar and gelatin media. The procedure is the same as for beef-extract media except that beef extract is not added.

Carbohydrate Broths.—To prepare these, 1 per cent of the various pure carbohydrates is added to plain beef-extract broth.

[5] The medium may be boiled, if necessary over the free flame; in this case it should be constantly stirred and brought back to original weight after it has been dissolved. It may also be dissolved by placing it in the Arnold steam sterilizer or even in the autoclav. With the latter apparatus a pressure of 10 or 15 pounds should dissolve the agar-agar in about thirty minutes.

[6] A better method for adjusting the reaction of culture media is given in a later paragraph in this chapter. This method requires the use of a comparator and carefully prepared standards.

This gives what are known as lactose broth, dextrose broth, inulin broth, etc. Oftentimes, an indicator is added to these carbohydrate broths to permit observations on reaction to be more easily made. For instance, Baker reported that brom-thymol blue could be added to such media before tubing and sterilizing; 15 c.c. of a 0.04 per cent alcoholic solution of the indicator to a liter of carbohydrate broth are sufficient. This makes it possible to follow changes in reaction as incubation progresses.

Several publications have reported the possibility of hydrolysis of the complex sugars to simpler ones if sterilized in the presence of proteins or certain protein derivatives. If a worker, for instance, used a medium which was supposed to contain sucrose, and if the sucrose was inverted or partly inverted during sterilization, his data would be secured not with sucrose but with its decomposition products. Consequently, in work demanding the best methods, the sugar should be sterilized separately in a water solution and the broth or agar in another container. The sterile sugar solution may then be added to the agar or broth in amounts to yield the desired concentration.

Nitrate Broth.—This medium is used for testing for nitrate reduction. It is prepared by adding 0.1 per cent of potassium nitrate to plain broth.[7]

Malt Broth for Yeasts.—Dissolve 15 grams of Difco dehydrated malt-extract broth in 1000 c.c. of water, distribute into containers and sterilize.

Stiritz Malt Medium for Yeasts.—Dilute 400 c.c. of so-called "near beer" with 600 c.c. of water. To each 100 c.c. of this medium, sterilized separately in a flask, add 4 c.c. of a sterile 5 per cent solution of lactic acid.

Plain Milk.—Fresh skimmed milk is placed in the container, plugged and sterilized. Plain milk may also be made by using 130 grams of skimmed milk powder per 1000 c.c. of water.

Litmus Milk.—Fresh, skimmed milk is colored with litmus, placed in any amount in either tubes or flasks, plugged and sterilized.

Brom-Cresol-Purple Milk.—This is plain skimmed milk to which the indicator brom-cresol-purple has been added. Many

[7] A longer list of media may be found in the author's "Bacteriology and Mycology of Foods," John Wiley & Sons, Inc., New York; the publications of the Digestive Ferments Co., will also be found helpful.

regard it as more satisfactory than litmus milk. This indicator changes color at the same hydrogen-ion concentration as litmus and has the advantage that it will withstand sterilization.

Whey Broth.—Add a little acetic acid to boiling milk until all of the casein is precipitated. Strain through cloth and neutralize or adjust the reaction of the filtrate to about *p*H 6.8. Add 1 per cent of peptone, tube and sterilize.

Synthetic Media.—Synthetic media are those prepared from pure salts. They contain no organic matter and possess the advantage that they may be duplicated, perhaps, more easily than media containing organic matter. One formula will be given below.

USCHINSKY'S MEDIUM (SMITH'S MODIFICATION, 1905)

Distilled water	1000	c.c.
Ammonium lactate	5	grams
Sodium asparaginate	2.5	grams
Sodium sulfate	2.5	grams
Sodium chloride	2.5	grams
Dipotassium phosphate	2.5	grams
Calcium chloride	.010	gram
Magnesium sulfate	.010	gram

LIQUEFIABLE SOLID MEDIA

Such media are those which may be liquefied and cooled to about 45° C., before inoculation; they become semi-solid at the ordinary temperatures of incubation. Robert Koch was the first to introduce gelatin into plain broth to make the gelatin medium which is in common use to-day. The introduction of this medium into bacteriological technic was one of the most epochal achievements for, very soon after, the causal organisms in many common diseases were discovered in Koch's laboratory. After this time the possibility of obtaining pure cultures was much greater, and bacteriologists could work with greater security and precision than before.

Beef-extract Agar.—This medium is quite similar to beef-extract broth, differing only in the fact that it contains some substance which will give a semi-solid medium. It has the following composition:

Beef-extract	3 grams
Peptone	5 grams
Distilled water	1000 c.c.
Agar	15 grams

Procedure:

1. Carefully clean the media pot supplied.

2. Put therein 1000 c.c. of distilled water, 5 grams of peptone, 3 grams of meat extract, and 15 grams of agar. Boil with constant stirring or cook in the autoclav until all of the ingredients are thoroughly dissolved. This may take fifteen or twenty minutes.

3. Adjust the reaction to neutrality by adding strong sodium hydroxide, using brom-thymol-blue. The same procedure may be used here that was suggested for the adjustment of reaction in beef-extract broth.

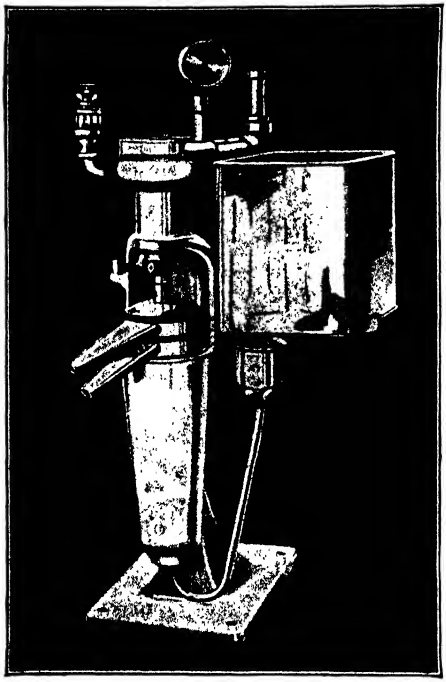


FIG. 27.—Laboratory Centrifuge for Clearing Media.

The hot medium is placed in the large receptacle at the right and flows by gravity through the centrifuge.

4. Filter to clear. This is not an easy task where special equipment is not available. Filtration may be accomplished by means of cotton in hot water-jacketed funnels. Instead of using such an apparatus, a Sharples Laboratory Centrifuge is used. Deliver your media to the buckets provided in the preparation room. The person in charge will filter and sterilize it.

In laboratories where a centrifuge is not available it is often an unsatisfactory task to clear the medium. A person has to use his ingenuity and arrive at the procedures that give him the best results. One method is to use a layer of cotton on a piece of cheesecloth in a large funnel. This will yield a clear medium after the filter has become covered to a slight extent with sediment from the medium. A water-jacketed funnel may be used to keep the medium from becoming too cold. This procedure frequently causes a loss of some of the medium since it is impossible to press all of it from the cotton.

Some of the older methods for clearing media advised the use of egg white, or even a whole egg, as a coagulant or clearing agent.

After the ingredients of the medium were dissolved, the temperature was lowered and the egg stirred in. Then the temperature was raised and, as the egg proteins coagulated, the factors responsible for turbidity were removed. This is not good practice since it is known that the egg contributes materials to the medium. Those who use egg in this manner prepare an egg agar, egg gelatin, etc. They do not secure a standard agar or gelatin medium.

Litmus Lactose Agar.—This is plain beef-extract agar to which has been added 1 part of azolitmin and 10 parts of lactose. It is especially useful for the detection of acid-forming bacteria.

Nitrate Agar.—Add 0.1 per cent potassium nitrate to plain agar.

Wort Agar for Yeasts.—Prepare a wort agar, using 15 grams of agar, 600 c.c. of distilled water and 400 c.c. of any of the so-called "near beers." The bottles should be opened and the contents poured into a beaker and boiled to rid the fluid of carbon dioxide. Thoroughly dissolve the agar, tube and sterilize in the usual way. This medium should be sterilized in known quantities in plugged containers. When ready to use it, melt in the sterilizer and to each 100 c.c. add 4 c.c. of sterile 5 per cent lactic acid. The acid must not be added to the agar before sterilization, else its jellying power will be destroyed.

Malt Agar Difco.—Dissolve 15 grams of Difco dehydrated malt extract in 1000 c.c. of water, add 15 grams of agar, dissolve, distribute into containers and sterilize. If sterilized twenty minutes at 15 pounds pressure, the *p*H should be 4.65.

Lactic Acid Dextrose Agar for Yeasts.—

1. Prepare dextrose agar in the usual manner outlined above; fill into Erlenmeyer flasks in 100 c.c. or 50 c.c. quantities. Plug and sterilize.

2. Prepare a 5 per cent water solution of lactic acid and sterilize in 10 c.c. amounts in test tubes.

3. When desired melt a flask of the dextrose agar and, with a sterile pipette, add 4 c.c. of the lactic acid solution to 100 c.c. of dextrose agar. Shake and distribute into sterile plugged culture tubes, plates, flasks, etc.[8]

Malt Syrup Agar.—Malt syrup sold in tin cans may also be used for the preparation of a medium for yeasts. The jellying power of the agar may be ruined if the malt is added to the agar

[8] This will yield a medium which will allow good growth of yeasts but not of bacteria. The acid must not be added to the agar before sterilization. Great care must be used in the manipulations to prevent contamination.

before sterilization. To avoid this the two solutions may be prepared separately as follows:

Solution 1.

500 c.c. water
3 grams malt syrup

Solution 2.

500 c.c. water
20 grams agar
5 grams peptone
3 grams of meat extract

Both of these solutions should be prepared and sterilized in separate containers. When malt agar is desired, equal amounts of each medium should be mixed, with sterile apparatus. If slants are desired the proper amounts of solutions 1 and 2 may be mixed in a sterile flask; from this flask the required amount of medium may be taken with a sterile pipette and delivered to a sterilized plugged culture tube.

Gorodkowa's Agar Medium for Yeasts.—This medium was proposed for showing the presence of ascospores in yeast cells. It has the following composition.

Distilled water	100	grams
Agar	1	gram
Meat extract	1	gram
Sodium chloride	0.5	gram
Glucose	0.25	gram

This medium may be slanted or used in Petri dishes. It stimulates the production of ascospores after two or three days.

MANEVAL'S MODIFICATION OF GORODKOWA'S AGAR MEDIUM

Distilled water	100	c.c.
Liebig's beef extract	0.3	gram
Sodium chloride	0.5	gram
Dextrose	0.25	gram
Agar	1.5 to 2	grams

Sterilize at 15 pounds pressure.

Beef-extract Gelatin.—This is prepared by adding 120 grams of gelatin to the beef-extract broth already prepared. (120 grams of gelatin should be used if Gold Label Brand is used, but only 100

grams if some such gelatin as Bacto-gelatin of the Digestive Ferments Company is available.) Some brands have greater jellying powers than others. The beef-extract gelatin has the following composition.

Water	1000 c.c.
Beef extract	3 grams
Peptone	5 grams
Gelatin (Bacto-gelatin)	100 grams

Procedure:

1. Clean the media pot which is in the locker.

2. Place therein 1000 c.c. of distilled water, 3 grams of meat extract, 5 grams of peptone and 100 grams of gelatin. Heat carefully until all of the ingredients are dissolved. The heating must be conducted carefully with gelatin, else its jellying or solidifying properties will be destroyed. Heating is best conducted in a double boiler or over a free flame with constant stirring.

3. Adjust to neutrality, using brom-thymol-blue as the indicator. This may be done in the usual manner as outlined in the preparation of plain broth.

4. The gelatin should be put into clean test tubes, plugged with cotton and sterilized. As soon as it is removed from the sterilizer, place it in cold water and then in the refrigerator.

Plain Gelatin.—Proceed as for beef-extract gelatin, but omit beef extract and peptone.

Carbohydrate Gelatin.—In the same manner as carbohydrate agars are prepared, carbohydrate gelatins may be prepared by adding 1 per cent of the sugars to plain gelatin.

Solid Media

Potato Slants.—Secure a large, evenly shaped potato and wash thoroughly. Peel and wash again. Then, by means of a cork borer of large size, make as many plugs as possible. These cylinders of potato should then be sliced obliquely and the two halves placed in culture tubes with the largest end downward. If the potato plug is suspended on a short piece of glass rod, a little water may be placed in the bottom of the tube to prevent drying of the medium during incubation. An alternative method is to place large discs of potato in the bottom of Petri dishes and sterilize in the autoclav.

Carrot Slants.—These may be prepared in the same manner as potato slants.

Dehydrated Media.—Composite media made according to standard formulæ are now available in the dehydrated state. Bacteriologists may quickly prepare these media by restoring the water to a weighed amount of the powder. After thorough solution, a finished, cleared, adjusted medium is obtained. This makes it possible for even the smallest laboratory to have all of the special media which, under some conditions, might be difficult to secure. The advantages of such media are apparent. Absolute uniformity is possible, and even small amounts may be prepared when only a few tubes are needed in the laboratory. The following, among others, are available:[9]

Bacto Nutrient Agar	*Bacto Eosine Methylene Blue Agar*
Bacto Nutrient Gelatin	*Bacto Endo's Agar*
Bacto Nutrient Broth	*Bacto Russell's Double Sugar Agar*
Bacto Dextrose Broth	*Bacto Lead Acetate Agar*
Bacto Lactose Broth	*Bacto Brilliant Green Bile*
Bacto Litmus Milk	*Bacto Nitrate Agar*
Bacto Purple Milk	*Bacto Peptonized Milk*
Bacto Wort Agar	*Bacto Corn Meal Agar*

And numerous other special media.

Filling Tubes with the Media.—While in the larger laboratories this is usually done by a technician, the student should have some experience. Various apparatus are available. The simplest may be made from a funnel fitted with a piece of rubber tubing and a pinchcock. Such an apparatus is shown in Fig. 28.

It is often desirable to deliver known amounts of media into tubes. Among others, those shown in Fig. 29 are available for this purpose.

Reaction of Media and Cultures

Besides the necessary food materials which have been mentioned above, culture media must have the proper reaction (must

[9] These dehydrated nutrient media are manufactured by the Digestive Ferments Company of Detroit, Michigan. This company publishes a very useful set of bulletins explaining the use of each medium. These will be found to be of great use for advanced students. A set of these bulletins should be available for reference in courses in introductory bacteriology. A booklet entitled "A Manual of Dehydrated Culture Media and Reagents" has also been published by this company.

be neutral or acid or alkaline). All forms of living organisms seem to react very markedly to acid or alkaline environments. This is probably due to the fact that protoplasm in living beings is in a delicate state of equilibrium and thus is very sensitive to such conditions in its environment. Consequently, a bacteriologist has to give some attention to the condition of his medium as far as acid and alkaline reaction is concerned.

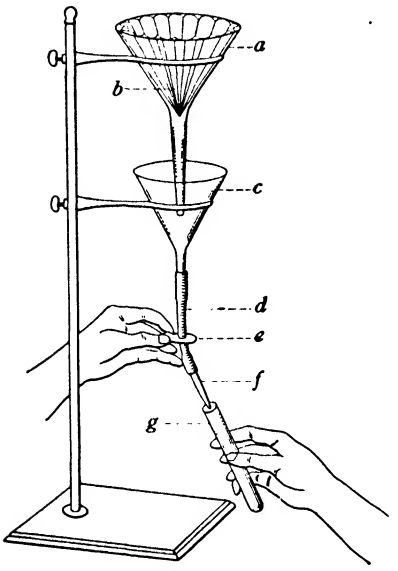


FIG. 28.—An Apparatus for Filling and Filtering Culture Media. (After Heinemann.)

a. Filter
b. Large funnel
c Small funnel
d. Rubber hose
e. Pinchcock
f. Pipette
g. Culture tube.

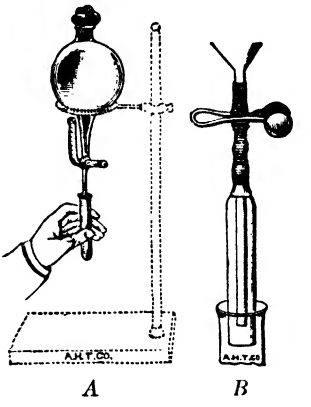


FIG. 29.—Culture Tube Filling Attachments.

A, For measuring exact quantities of fluids. *B*, For use in filling culture tubes, etc. This device prevents contact of the fluid with the surface of the vessel being filled so that the latter remains clean for the insertion of the cotton plug.

Indicators.—An indicator is a pure chemical compound which, by changing color, will "indicate" either an acid or an alkaline condition. The indicators themselves are changed. The best known indicator is, perhaps, litmus, which is available either as a liquid or as litmus paper. Litmus turns blue in the presence of alkali and red in the presence of acid. In order to qualify these terms, such expressions as "faintly acid," "strongly acid," etc., have crept in. These are terms with widely different meanings

and, as Taylor stated, are better replaced by the hydrogen-ion concentration designation, such as 7.4, 7.6, etc. The following table [10] will show the relation between the older expressions for acidity and the hydrogen-ion concentration.

TABLE V

Acidity	Indicator Reactions	Approximate *p*H Value
"Neutral"	Blue or Green to Brom-thymol Blue	Over 6 2
"Weak"	Yellow to Brom-thymol Blue Purple to Brom-cresol Purple	5 2-6 0
"Moderate"	Yellow to Brom-cresol Purple Orange to Methyl Red	4 6-5.0
"Strong"	Maximum Red to Methyl Red Blue or Green to Brom-phenol Blue	3 2-4.4
"Very Strong"	Yellow to Brom-phenol Blue	Under 3 0

How is Reaction Expressed?—Different methods have been used for expressing the reaction of media. It has been expressed as per cent acidity or alkalinity, each cubic centimeter of normal acid or alkali per 100 c.c. of medium being equivalent to 1 per cent. Reactions are also expressed by Fuller's scale in degrees. Each cubic centimeter of normal acid or alkali in a medium is equivalent to a degree. A medium adjusted to 1 per cent acid (containing 10 c.c. of normal HCl per liter) would, according to Fuller's scale, be 10 degrees acid.

Titration Methods.—Such methods were used by bacteriologists prior to the more common colorimetric hydrogen-ion concentration methods which are in use to-day. Reactions were expressed in terms of cubic centimeters of normal hydrochloric acid or sodium hydroxide per liter. The procedure of titration was as follows:

1. Place 5 c.c. of the medium to be adjusted in a casserole and add 50 c.c. of distilled water.
2. Bring to boil and add several drops of phenolphthalein indicator.

[10] From "Manual of Methods for Pure Culture Study of Bacteria," Committee on Bacteriological Technic of the Society of American Bacteriologists.

3. Titrate with N/20 NaOH to the point where a faint pink color is permanent.

4. Compute as follows: Assume that 2.4 c.c. of N/20 NaOH were required. 5 c.c. of medium required 2.4 c.c. N/20 NaOH. 1000 c.c. would require 480 c.c. N/20 NaOH. 480 c.c. N/20 NaOH = 24 c.c. N NaOH.

This means that the medium would require 24 c.c. of N NaOH for neutralization. If it is desired to adjust it to 1 per cent alkaline, 34 c.c. of N NaOH will have to be added.

Titration with phenolphthalein as the indicator was the method formerly used by bacteriologists for adjusting the reaction of culture media. This method was based on assumptions which were incorrect. Clark and Lubs' work indicated that the hydrogen-ion concentration should be determined. Phenolphthalein did not indicate the true neutral point. Furthermore, the alkali which was added to neutralize did not always indicate its reaction. Titration methods are not without value. They give information with regard to the "buffer index" of a medium.

Measurement of Hydrogen Ion Concentration.—In general, two methods may be used for measuring the hydrogen-ion concentration; the first is the electrometric method; and the second, the colorimetric method.

The Electrometric Method.—This requires the use of expensive, delicate apparatus. Considerable time is also consumed in making a determination. On account of these facts, the colorimetric method was devised. The electrometric method, however, is necessary for the standardization of the indicators and buffer solutions to be used in the colorimetric method.

What is Meant by Hydrogen-ion Concentration or pH?—This is discussed by Taylor of the La Motte Chemical Products Company. It may be helpful to reproduce his discussion.

"Everyone is familiar with the Fahrenheit thermometer. On this scale, 32° represents the freezing point of water. For the sake of illustration we shall assume that values above and below 32° represent degrees of heat and cold respectively. Thus, any values higher than 32°, such as 34°, 36°, 40°, etc., denote an increase in heat, the degree of heat *increasing* as the numbers *increase*. On the other hand, any values below 32°, such as 30°, 28°, etc., denote an increase in coldness, the degree of coldness *increasing* as the numbers *decrease*.

"In an exactly similar manner the degree of acidity or alkalinity of a solution is expressed by the hydrogen-ion scale. Instead of being called degrees, as in the case of the thermometer, the units on this scale are called *p*H values. It is apparent that it is not necessary for a person to know the

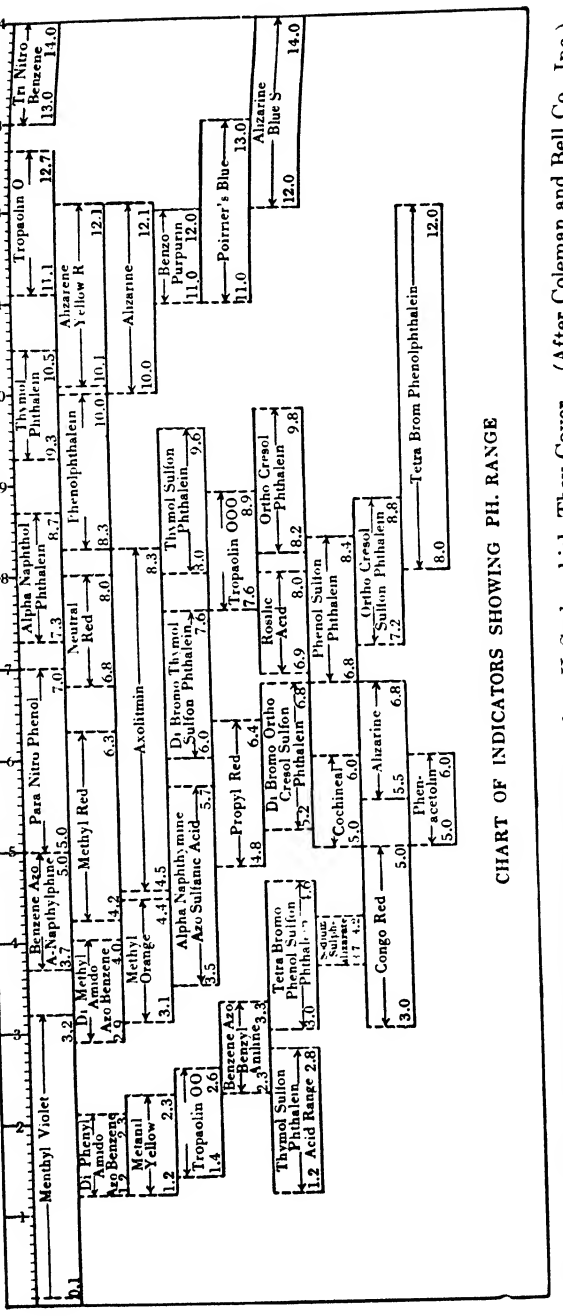


FIG. 30.—Chart of Indicators Showing the Range on the *p*H Scale which They Cover. (After Coleman and Bell Co., Inc.)

derivation of the term "degree Fahrenheit" in order to determine the temperature of a solution. It is equally true that the worker need not know the meaning of the term "*p*H" in order to use this method for measuring acidity and alkalinity. On the hydrogen-ion scale, a value of *p*H 7.0 represents neutrality. This means that if the material being tested has a *p*H of 7.0 it is neither acid nor alkaline.

"Following the illustration of the thermometer given above, any values higher than *p*H 7.0, such as 7.2, 7.4, 8.0, 9.0, 10.0, etc., denote alkalinity, the degree of alkalinity increasing as the numbers increase. Analogously, any values lower than *p*H 7.0, such as 6.8, 6.6, 6.0, 4.0, 2.0, etc., denote acidity, the degree of acidity increasing as the numbers decrease. This will be clear from the following tabulation of *p*H values.

Acid	Neutral	Alkaline
←		→
etc. 5.6, 5.8, 6.0, 6.4, 6.6, 6.8,	7.0,	7.2, 7.4, 7.8, 8.0, etc.

"Since the significance of '*p*H' has been explained, we shall use this term throughout to denote acidity and alkalinity. It is apparent that if a solution has a *p*H of 7.6 it is alkaline. If another has a *p*H of 8.2 it is more alkaline than the one of *p*H 7.6. It is also clear that acid must be added to a solution of *p*H 7.6 to bring it to the neutral point of *p*H 7.0 and that larger quantities must be added to bring it to an acidity of 6.8, 6.0, etc. Similarly, if a solution has a *p*H of 6.0, it is acid, and it is necessary to add alkali to it in order to bring it to the neutral point *p*H 7.0, or to an alkaline value such as *p*H 8.0.

"In order that the worker may have some idea of the degree of acidity corresponding to various *p*H values, it may be stated that a solution which has a *p*H value of 5.0 is ten times as acid as one with a *p*H of 6.0. Analogously a solution of *p*H 4.0 is ten times as acid as one of *p*H 5.0. Thus *p*H 4.0 indicates an acidity 100 times as great as *p*H 6.0. A similar relationship holds on the alkaline side of the scale. That is, a solution which has a *p*H of 9.0 is ten times as alkaline as one which has a *p*H value of 8.0, etc."

The Colorimetric Method.— This method requires the use of indicators, chemical compounds which have one color in acid solution and another color in alkaline solution. However, it is necessary to know just where these indicators change color on the hydrogen-ion scale. This must be determined by the electrometric method and, once it has been determined for a chemical compound, the change of color of that substance may be accepted as indication of the different hydrogen-ion concentrations for which it is sensitive.

Until about 1916 litmus and phenolphthalein were the commonest indicators used. Litmus is not a delicate indicator because it is not a definite chemical compound. The active constituent is azolitmin. The real reason for the inadequacy of

litmus as an indicator is its wide range (*p*H 4.6–8.4). The color changes are not distinct. Phenolphthalein, while it does not possess some of the disadvantages of litmus, possesses others which influence its action as an indicator in bacteriological work. Its action is influenced by carbon dioxide, a product which is frequently present in bacteriological material. The end point is not sharp.

The unsatisfactory state of knowledge on this subject stimulated investigation, and the work of Clark and Lubs has given us some new chemical compounds which may be used as indicators in bacteriological work. These are shown in Table VI.

TABLE VI

THE NEWER INDICATORS FOR BACTERIOLOGICAL WORK *

Indicator	Full Acid Color	Full Alkaline Color	Sensitive Range
Thymol blue (acid range)	Red	Yellow	1.2–2.8
Brom-phenol blue	Yellow	Blue	3.0–4.6
Brom-chlor-phenol blue	Yellow	Blue	3.2–4.8
Brom-cresol green	Yellow	Green	3.8–5.4
Methyl red	Red	Yellow	4.4–6.0
Chlor-phenol red	Yellow	Red	5.0–6.6
Brom-cresol purple	Yellow	Purple	5.2–6.8
Brom-phenol red	Yellow	Red	5.4–7.0
Brom-cresol purple	Yellow	Purple	5.2–6.8
Brom-thymol blue	Yellow	Blue	6.0–7.6
Phenol red	Yellow	Red	6.8–8.4
Cresol red	Yellow	Red	7.2–8.8
Thymol blue (alkaline range)	Yellow	Blue	8.0–9.6
Phenol phthalein	Colorless	Red	8.0–9.6
Cresol phthalein	Colorless	Red	8.2–9.8

*A longer list of indicators will be found in the Appendix. This list was taken by permission from the catalogue of the British Drug House, Ltd., of London.

Action of Buffers, "Buffer Index."—The bacteriological materials, the reaction of which have to be determined, are never solutions of pure acids or alkalies. For instance, in plain broth there are peptones, inorganic salts, etc. These exert what is called a "buffer action," and these materials are themselves

called " buffers." They may be defined as materials that resist changes in *p*H through the addition or loss of acid or alkali. The " buffer index " of a substance is the sum of the reserve alkalinity and reserve acidity.

Method for Adjusting the Reaction of Culture Media.—This procedure is founded on Barnett and Chapman's method. (Standard Methods of Milk Analysis.)

1. Select twelve test tubes which have been graded for size, clean, dry and place in a test-tube rack in two rows of six each.
2. In each tube of one row place 5 c.c. of dilute alkali, such as N/20 NaOH.
3. In each tube of the other row place 5 c.c. of dilute acid (1 drop of concentrated sulfuric or hydrochloric acid to 100 c.c. of water).
4. Add indicator (brom-thymol blue) as follows:

Acid Tubes, Drops	Alkali Tubes, Drops	Hydrogen-ion Concentration
9	1	*p*H = 6.2
8	2	6.4
7	3	6.7
6	4	6.9
5	5	7.1
4	6	7 3

5. View the tubes in pairs of acid and alkali, each pair containing the sum of 10 drops of indicator.
6. Put 4 c.c. of distilled water in a test tube and add 1 c.c. of medium to be tested (agar, broth, etc.), and then 10 drops of brom-thymol blue. Compare with sets of standard indicators prepared.
7. The reaction of the agar should be between *p*H 6.8 and 7.0. If the correct color of the medium does not appear in the agar tested, add dilute N/20 NaOH from a burette until the correct shade is secured, indicating a reaction between 6.8 and 7.0. Fifty times the amount of N/20 NaOH added from the burette indicates the amount of N NaOH which must be added to adjust 1 liter of the agar.

Another rough method may be used by those who cannot use the method just described. Drops of indicator may be placed in the depressions of a test plate and drops of agar medium added as the reaction is changed. The neutral point for brom-thymol blue is grass-green. The reaction of the entire pot may be brought to a grass-green for neutrality, a color chart being used for comparison.

Taylor outlined the following procedure for making a rough

test to find out what indicator must be used, when the worker has no idea of the *p*H of a given solution.

1. Fill three or four graduated test tubes to the 10-c.c. mark with the solution to be tested.

2. To the first test tube add 0.5 c.c. of brom-thymol blue to determine

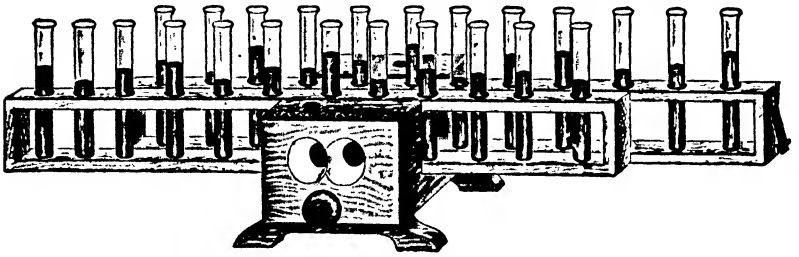


FIG. 31A.—The Cooledge Comparator for the Comparison of a Series of Unknowns with a Graduated Series of Standards for the Determination of Hydrogen Ion Concentration. (*Central Scientific Company, Chicago.*)

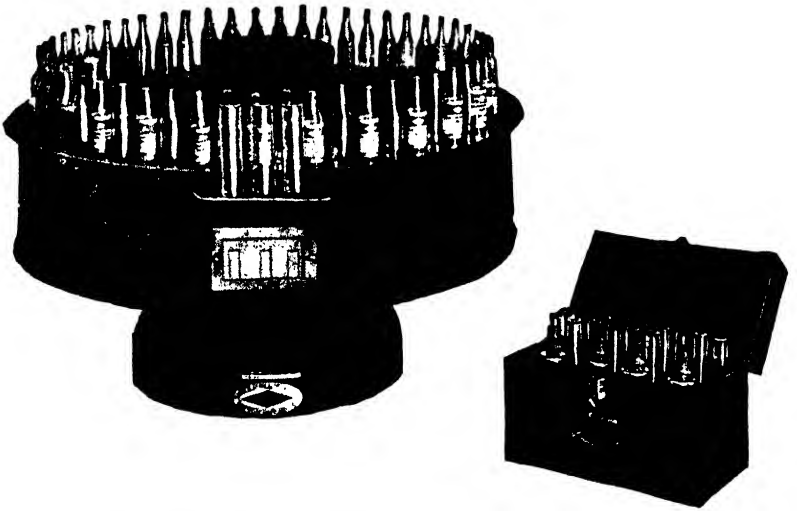


FIG. 31*B*.—Roulette Comparator. (*La Motte Chemical Products Co.*)

whether the solution is neutral, acid or alkaline. If the color is intermediate between yellow and deep blue, the true *p*H is within the limits of 6.0 and 7.6. No further rough tests are necessary.

3. If a yellow color is obtained, the *p*H is at least 6.0 and possibly lower. Add 0.5 c.c. of brom-cresol green (*p*H 4.0–5.6). If the color is between a yellow and a deep blue, no further rough tests are necessary with this indicator.

4. If a yellow color is secured with brom-cresol green, the procedure should be repeated with brom-phenol blue, and so on.

In the author's laboratory the " Roulette Comparator " made by the LaMotte Chemical Products Company of Baltimore, Maryland, is used. Other comparators are probably just as satisfactory but may not be as convenient. The following steps are followed for adjusting the reaction of culture media.

Procedure.—1. Select three clean test tubes and fill to the mark 10 c.c. with the medium (plain broth, plain agar,* plain gelatin, etc.) to be tested.

2. Place them in the three holes in the block. Add 0.5 c.c. of brom-thymol-blue to the middle tube of medium and shake to insure thorough mixing.

3. Turn on the light and revolve the drum until the brom-thymol-blue color standards are directly behind the test samples in the block.

4. Looking toward the light through the three slots, slowly shift the standards by revolving the drum until the color through the central tube exactly matches that of one of the tubes on either side of it, or lies between them. The *p*H value can then be read from the standards. The reaction should be *p*H 7.0.

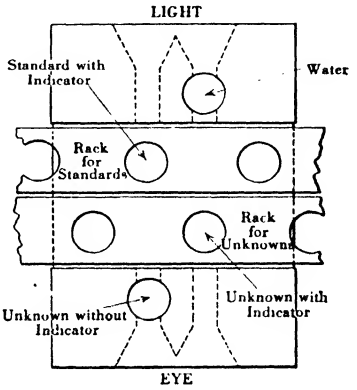


FIG. 32.—Showing the Location of the Tubes in the Cooledge Comparator. (*The Central Scientific Co., Chicago.*)

5. If too acid as it usually is (indicated by a *p*H reading of 6.6., 6.8, etc.) add a small amount of normal sodium hydroxide or a strength close to this value to the media in the pot, stir, and test again. If desired the alkali may be run from a burette and the exact amount of sodium hydroxide to be added computed.

Sterilization of Media.—One of the prerequisites of a good medium is sterility. The methods of sterilization have been discussed in Chapter I. It is well to point out that tubes of culture media should not be packed too tightly in baskets nor should the autoclav be packed full of baskets. Some free space should be available for circulation of the steam. Oftentimes, a preparation room may become infected with a resistant spore-forming organ-

* When agar medium is used it should be diluted about 1 to 5 to keep it in the liquid state.

ism which may survive the time and temperature then being used for sterilization. At such times, it may be necessary to use longer times and higher temperatures for sterilization.

Storage of Media.—Culture media should not be kept longer than absolutely necessary. They evaporate sufficiently to change their composition and may also become contaminated. Such media as must be kept should be stored in the refrigerator. Special media, difficult to prepare, which may be prepared in larger amounts, may be stored in flasks which have been sealed. The flasks may be drawn out before filling and plugged with cotton after filling, just before sterilization. The flasks may be tipped after sterilization and stored. Needless to say, it is extremely important that stored media be carefully labeled.

REFERENCES ON MEDIA

Digestive Ferments Co., Detroit, Michigan. Special bulletins on various prepared culture media; also, Manual of Dehydrated Culture Media and Reagents, 1927.

EYRE, J. W. The Elements of Bacteriological Technique. W. B. Saunders Company, Philadelphia, 1916.

MCCRUDDEN, F. B. The Determination of Hydrogen Ion Concentration, Public Health Repts. 37 (1922), 334–348.

The Reaction of Media. Medical Research Committee, Special Report Series No. 35, Medical Research Council. His Majesty's Stationery Office, 1927.

Standard Methods for the Examination of Water and Sewage. American Public Health Association, 1920.

TANNER, F. W. The Bacteriology and Mycology of Foods. John Wiley & Sons, Inc., New York, 1919.

TAYLOR, W. A. The A B C of Hydrogen Control. La Motte Chemical Products Company, Baltimore, Md., 1926.

CHAPTER IV

MICROSCOPIC EXAMINATION OF BACTERIA

THE small size of bacteria makes it necessary to use a microscope for their careful study. They may be examined in either the stained or unstained condition. Several magnifications may be used. However, for continued success and comfort, the oil-immersion objective with an ocular giving a magnification of about one thousand will be most satisfactory. Other combinations giving higher magnifications are available for special work.

The student will find it profitable to heed the advice given in the discussion on the use of the microscope. If the microscope is not properly used, good results from the examination of stained and unstained preparations cannot be expected.

EXAMINATION IN UNSTAINED PREPARATIONS

These preparations permit the examination of bacteria without the addition of stains. Consequently, the cells are not altered to any great extent and, especially in the hanging drop, are free to move about.

Hanging Drop Preparations.—These preparations differ from the stained mounts in that the unstained bacteria are free to move about. Different characteristics of the organisms may be determined from the hanging drop. The apparatus involved may differ in details of construction but essentially it is the same. The bacteria are suspended in a drop of water in or over a depression of some sort. Concave well slides are used, or ordinary plain slides bearing a rubber or glass ring [1] are equally satisfactory.

[1] Rubber rings about 1 cm. in diameter and 2 or 3 mm. high are available from supply houses. These may be fastened to glass slides with different materials as vaseline, paraffin, etc. A recent writer advised the quick drying lacquers that have been recently developed, for this purpose. Avoid the use of too much cement and thus have a neat slide.

1. Clean the concave slide or ring slide and cover glass.

2. Place a drop of sterile water in the center of the cover glass and transfer a little of the culture to it.

3. Place a small amount of vaseline around the edge of the cover glass. Do not use too much. Just enough should be used to seal the preparation when placed over the depression on the slide.

4. Place the cover glass over the depression and gently press to spread the vaseline out on the slide or ring. (See illustrations.)

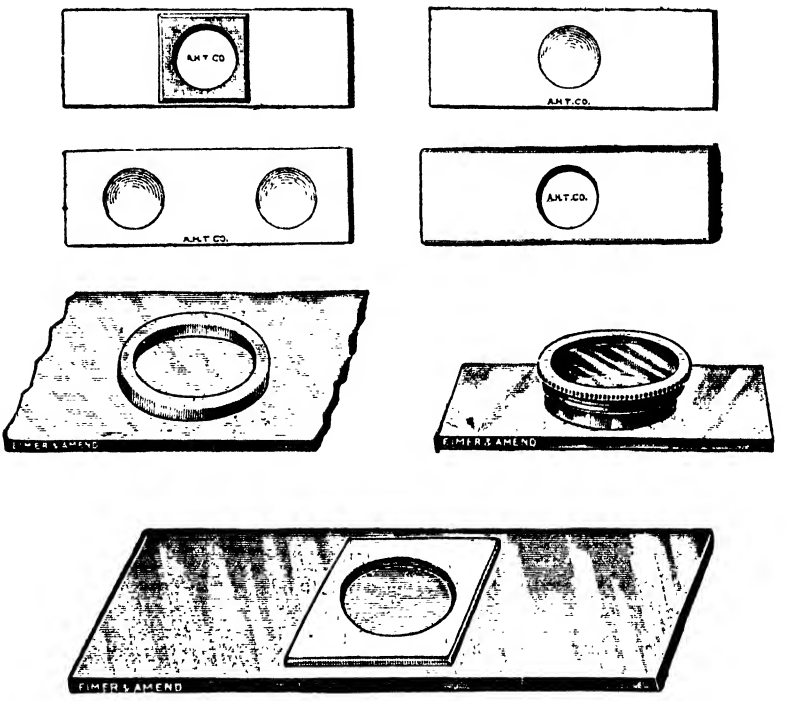


FIG. 33.—Types of Concave Slides and Moist Chambers for Making Hanging Drop and Hanging Block Mounts. (See also Fig. 8.)

Lindner's Adhesion Culture.—This method consists of spreading the culture liquid in a thin layer on the underside of a cover slip and fixing the latter with vaseline on a hollow slide or ring. In this way the whole field of view is practically in one plane. In droplet cultures the individual cells or cultures have too much nourishment, and the multiplication is too intense. In the adhesion culture the cells are spread with such a thin layer of liquid that they are held fast in one place all of the time by adhesion to

the cover slip. Since the supply of nutriment in any one part is strictly limited, the multiplication is relatively slow; consequently the colonies grow out in a circular form with the original mother cell in the center.[2] There is a sufficient quantity of air enclosed in the chamber for development under fully aerobic conditions, and the adhesion culture is better than the gypsum block for inducing spore formation. If it is necessary to prepare adhesion cultures with restricted aeration the best way is to spread the liquid between two cover slips, of which the lower one is circular and very thin and small enough to go inside the hollow of the object slide, while the upper one is large enough to cover the hollow and to close it with a vaseline ring. In preparing adhesion cultures it is important not to use too much nutrient medium; the medium should generally be diluted. It is also essential to remove all traces of fat from the cover slips in order to secure a continuous film of fluid. A trace of fat on the glass is of advantage, however, in that it causes the drop to assume a globular form.

The chief difficulty with such preparations lies in the extreme thinness of the layer of liquid on the glass. If the hollow space on the slide be very dry the culture liquid may evaporate after the slip is mounted. It may, therefore, be advisable to breathe on the slide before applying the slip. Again, if the culture be unevenly heated after mounting, in the sense that the cover slip becomes warmer than the slide, the moisture will evaporate and condense on the lower part of the hollow; care should always be taken, especially during incubation, that the slide be kept warmer than the cover slip.

Hanging Block Mounts.—These require the same apparatus as the hanging drop. The bacteria, instead of being free to move about are fixed between the agar block and cover slip.

1. Pour a sterile agar plate, allow to cool and harden.
2. By means of a sterile spatula or knife, cut and lift out a block of the sterile agar and allow it to rest on the knife blade. (Block = 7 or 8 mm. square.)
3. Rub some of the culture on the surface.
4. Place the block on the cover slip in such a way that the bacteria are between the cover slip and the block.

[2] Lindner, P., The Adhesion Culture, A Simple Method for the Biological Analysis of Mixed Growths. Wochenschr. Brau., 18 (1901), 512–514; Jour. Fed. Insts. Brewing 7 (1901), 482–483.

5. Invert the glass cover slip carrying the agar block over the depression on the slide.

6. Incubate at the proper temperature and observe after a few hours.

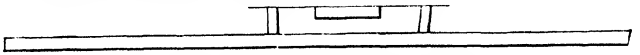


FIG. 34.—Hanging Block Preparation.

The bacterial cells are placed between the cover slip and the block of agar medium.

India Ink Preparations.—The ink used in this preparation is also known as Chinese ink.[3] The preparations are made as follows:

FIG. 35.—*Streptococcus cremoris*. Burri's India Ink Preparation. (After Orla-Jensen.)

1. Secure a tube of dilute India ink and do not shake or disturb.

2. Place a drop in the center of a clean slide.

3. By means of a sterile loop or needle, transfer a little growth or other material to the India ink and spread it over an area of 1 or 2 centimeters on a slide, and allow to become thoroughly dry.

4. Mount in Canada balsam for examination with the microscope.

Dark-field Illumination Examination of Unstained Microorganisms.—This method of examination causes the cells of the organism being examined to appear as bright refractile granules on a black background. The apparatus used is shown in Fig. 36. Instead of bringing the light up through the condenser in the usual manner, the dark-ground condenser permits only oblique rays to strike the object. The dark-ground condenser fits into the

[3] India or Chinese ink frequently contains living bacteria. These may cause some difficulty in using this material for the study of microorganisms. The ink secured from the supply houses should be sterilized in the autoclav and stored in small containers so that the stock supply will not have to be opened except to remove a supply to a smaller container. If the ink is kept over long periods of time it should be diluted with water and resterilized. Always use sterile apparatus when removing the ink to the slides or tubes. Hagan has reported that Grübler's ink, known as Pelikan Tusche No. 541, is useful and apparently contains a preservative since he has been unable to find bacteria in several samples.

substage of the microscope in the same way as the Abbé condenser. The Bausch & Lomb Optical Co. have described the apparatus required as follows:

"**Object Slide.**—Only glass slides ranging in thickness from 1.45 to 1.55 mm. should be used, in order that the illuminator may be brought into proper relation with the object. If too thick a slide is used the illuminator cannot be brought near enough to the object to focus properly, and upon looking at it a ring of light will appear in place of a very small bright point of great intensity. This occurs also if too thin a slide is used.

"**Objectives.**—*Achromatic objectives* are so perfect in their correction that they are especially suited for this class of investigation. A 4-mm. *achromatic*

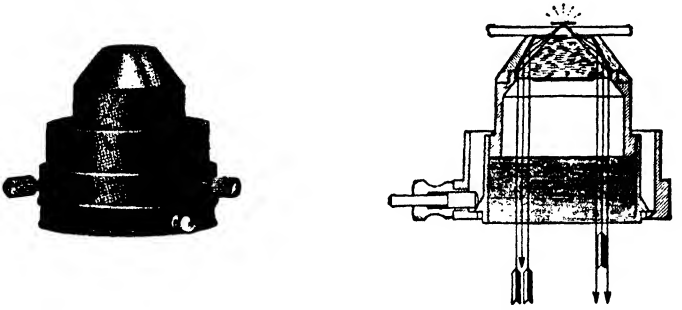


FIG. 36.—Showing the Apparatus used for Dark Ground Illumination.

Note how the light rays are brought on to the object from the sides. This gives a bright translucent object in a dark field. The image secured with this condenser is quite like that secured in Burri's India ink preparation.

objective 0.65 N. A. is recommended as being a lens which will be found well adapted for this work. If an *oil-immersion objective* is preferred on account of the higher magnification, a marginal stop should be inserted above the back lens in order to prevent direct rays from entering the objective. This may be done by unscrewing the rear portion of the objective mount, inserting a funnel stop, apex down, then screwing it again into position.

"**Cover Glass and Tube Length.**"—Objectives are corrected for a certain cover-glass thickness (B. & L. 0.18 mm.), and tube length (160 mm.). In order to secure the best results these conditions must be carefully observed.

"**Light Source.**—The light source should be powerful, such as direct sunlight, or electric arc, Nernst, acetylene, calcium or inverted incandescent gas lamps.

"**Manipulation.**—The *lens mount* should be screwed into the *adjusting sleeve* until its upper margin is flush with the top of the stage. The upper surface of the front lens of the illuminator bears a small index circle ruled centrally on the glass.

"In centering the illuminator it should be first viewed with a low-power objective and eyepiece, such as a 16-mm. objective and 7.5x or 10x eyepiece. Looking into the microscope and using the centering screws, the small circle

should be brought into the center of the field. This being accomplished, the illuminator is properly centered with reference to the objective and eyepiece.

"The light from the arc lamp, or other source of illumination, if artificial, should be made parallel by means of a condenser, and thus projected on the plane mirror of the microscope. The small hand-feed arc lamp is supplied with a bull's-eye condenser of suitable focus for this purpose. Attached to the lamp is a tube in which the condenser is adjustable.

"A glance at the top surface of the illuminator, before placing the object on the stage, will show a faintly illuminated ring (the reflection of light on the top surface of the illuminator), and another one of greater diameter will be seen on the upper surface of the opaque stop. When these rings appear perfectly round and central, the light is properly centered. This may be accomplished by altering the relative position of mirror and light source, and having the bull's-eye, or the globe condenser, so adjusted as to fill the mirror with light.

"The object, which must be mounted in fluid, may now be placed upon the stage with immersion oil or water between the bottom of the object slide and the illuminator. If the immersion fluid is omitted no illumination occurs. The illumination may be regulated by the iris diaphragm.

"Air bubbles in the oil must be carefully removed, if necessary, by the application of a hot needle, since their presence makes it impossible to obtain a perfectly dark field. We may add that for the same reason it is absolutely necessary to use slides and cover glasses which are perfectly clean and free from scratches."

EXAMINATION IN STAINED PREPARATION

Stained preparations have been used since the early days of the science and much progress has been made with them. They are used as bases for many diagnostic procedures. It should be pointed out, however, that stained bacteria, while perhaps not necessarily killed, are greatly altered in appearance.

Stains and Their Properties.—The number of dyes or stains for microbiological work is increasing every day. Each investigator in the science cannot hope to become proficient in the use of all stains and staining mixtures, but will probably find it advantageous to use a small number with which he secures the best results.

The chemical constitution of most of the common dyes is known. In the dyeing industry, it is known that a certain group, called the *chromophore* group, must be present. A *chromogen* is the simplest substance containing this chromophore group. The azo—$N = N$— group is one of the commonest chromophore groups. Since there are compounds containing this chromophore group which possess no ability to stain, it is assumed that another group, called the *auxochrome*, must be present before staining or

dyeing results. The phenol group is one of the common auxochrome groups. The amido group is another.

Stains known as aniline dyes are commonly used in bacteriology. For convenience, Ehrlich divided them into two groups, the basic and the acid dyes. The basic dyes contain an amido group. They possess a special affinity for nuclei and form the basis of nuclear stains. Usually these dyes are salts of hydrochloric and acetic acid. These basic dyes form the basis for all useful staining solutions in microbiology. Some of the more important ones may be classified as follows, according to the color which they impart to the cell:

Green: Malachite green, methyl green.
Red: Neutral red, safranin, fuchsin (basic) rubin.
Blue: Methylene blue, thionin blue, Victoria blue, quinoline blue, Azur, Nile blue.
Violet: Crystal violet, dahlia, methyl violet, gentian violet.
Brown: Bismarck brown.

The acid dyes are not used extensively in bacteriological work. They are characterized by the acidic OH group and are usually salts of sodium or potassium. They do not possess the specificity for certain tissues that obtains with the basic dyes, but stain all types of tissue less easily. They are used, however, to a certain extent as counterstains. Among the acid dyes are found eosin, magenta, fluorescein, aurantia, acid fuchsin, and picrocarmine. When these acid dyes are used to stain bacteria, they are used along with a mordant such as alum tannic acid, iron salts, iodine, etc.

The United States had not been long in the World War before bacteriologists found themselves in a critical situation as far as a supply of reliable stains was concerned. After the signing of the Armistice in 1918 it was apparent that study should be given to the problem and the Committee on Bacteriological Technic was induced to inaugurate such an investigation. Previous to this time bacteriologists had believed that they could not get along without a supply of Grübler dyes for biological work. In fact, it was generally believed that American dyes could not be used. Conn (1922) started the study of these materials which has resulted in the availability of a supply of dyes for bacteriological work which are superior and more even in chemical constitution than the older imported brands. A Biological Stain Commission has

been established and will place the study of these products on a permanent, dignified basis.

Mordants.—In the dyeing industry there are instances in which it is necessary to use an intermediate compound to bind the dye to the fabric. Such compounds are called mordants. They are also used for the staining of bacterial cells and probably serve here the same purpose that they serve in dyeing fabrics. These mordants increase the rapidity of staining and render the color of the stained cell more intense or deeper. Some common mordants used in biological work are as follows:

Iodine in potassium iodide
Bromine in potassium bromide
Tannin
Tannic acid
Phenol
Various acids
Various alkalies

Post (1922) has reported that a number of different reagents increase the staining power of certain of the aniline dyes. A number of acids and alkalies were used in this way.

Theory of Staining.—Different theories have at times been advanced to explain the staining process. Probably none of them entirely explains it.

According to some, the staining process may be explained entirely on a *physical* basis. In this theory, saturation of the cell with the dye would occur. The adherents of this theory claimed that when heat was applied, the interstices in the membrane of the cell were made larger, and the dye allowed to enter more easily.

The *chemical* theory holds that there is a true chemical reaction between the dye and the protoplasm of the cell. According to the supporters of this theory, heat acts in the same manner as in any chemical reaction—to drive it faster. The possibility of there being some chemical reaction is supported by the fact that the stain has to have a certain chemical structure before it may function in coloring the cell. The fact that the basic dyes are required in bacteriology for staining bacterial cells which are supposed to contain much nucleic acid also favors this chemical theory. Here there would be a reaction between the nucleic acids and the basic dye.

In the dyeing of textiles, the explanation has been made on a colloidal basis. This seems to be in keeping with the increase

in our knowledge of colloids. The colloidal explanation has some support in those procedures in staining that require a mordant. Unna (1888) has mentioned that these dyes are not always basic, but may be salts, neutral in character. They are called basic simply because the colored component is basic. The aniline dye does not separate into two parts, the basic or colored part attacking the cell protoplasm, but rather the entire molecule plays its part in the staining process. That the staining ability is deter-

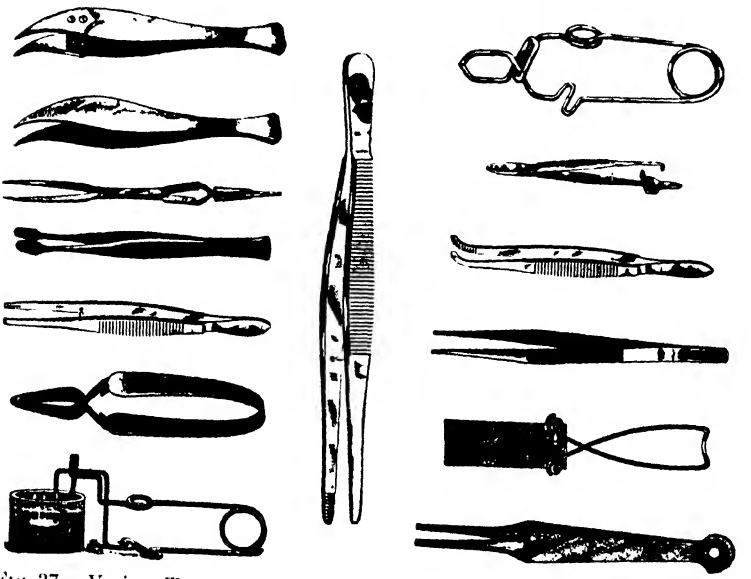


FIG. 37.—Various Types of Forceps Used in Bacteriological and Staining Work.

mined by the solvent condition of the dye is shown by the following observations.

1. Pure alcoholic water-free solutions of the dyes do not stain.
2. Pure absolute alcohol will not decolorize while alcohol diluted with water decolorizes quickly.
3. The more perfect the solution of the dye in the solvent, the less does its staining ability become. This helps to explain why absolute alcohol does not decolorize and why pure alcoholic solutions do not stain.

Preparation of Apparatus.—One of the most important essentials of the staining procedure is clean apparatus. All grease and foreign matter must be removed from the slides and cover glasses

according to the procedure outlined under cleaning apparatus in the first chapter. After the slides have been thoroughly cleaned they may be kept in a salt-mouth bottle in alcohol. The cover slips may be kept in a short wide-mouth bottle containing alcohol or ether. In this way both the slides and the cover glasses will be free from grease, provided they are thoroughly cleaned and rinsed in distilled water. While any method and apparatus may

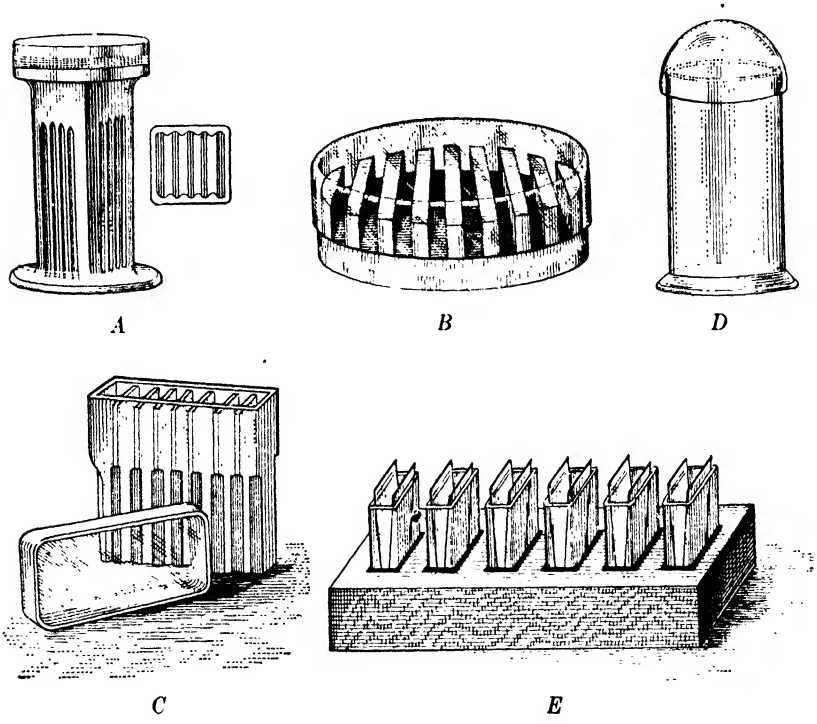


FIG. 38.—Types of Staining Jars and Dishes.

A, Coplin's; *B*, Moore's; *C*, Lellendahl's; *D*, Naples Jar; *E*, Series of Jars for Differential Staining.

be used to stain bacteria, certain types have been found to be more convenient than others. When the organisms are mounted on a slide, they may be covered with the stain or immersed in it. For immersion, many different types of staining jars are available. When the staining procedure is carried out by putting the dye on the bacteria on a slide or cover glass, it may be kept in ordinary dropping bottles.

Staining Solutions.—The bases for the staining solutions are the basic aniline dyes. These may be prepared in larger quantities and stored in the dark in glass-stoppered bottles. Aqueous solutions of these dyes are very weak stains, but they have the advantage that overstaining is unlikely when they are used. These alcoholic solutions of the dyes exhibit slight staining ability and are diluted with distilled water to give what are called the " aqueous alcoholic " stains. Generally, from 1 to 5 c.c. of the saturated alcoholic solutions are diluted to 100 c.c. with distilled water.[4] This yields a solution with the proper amounts of alcohol, water and dye to give best results. Much better results would be secured in many cases if these solutions were diluted still further.

Conn (1922), who has been giving considerable study to American stains, has called attention to two general types of stain formulæ. In one type the dye is expressed in grams per cubic centimeter while in the other type so many cubic centimeters of a saturated alcoholic solution of the dye are used. Since different lots of dyes contain different amounts of inert material, similar staining solutions may not be secured as would ordinarily be expected. Conn (1922) has, therefore, urged that bacteriologists use the formulæ requiring so many cubic centimeters of a saturated alcoholic solution of the dye. An aqueous solution may be used when specified.

Preparation of Film for Staining.—This is an important step in the microscopic examination of bacteria and should be performed with care. A good film will not have too many bacteria but will have a sufficient number to allow accurate observations. It is prepared as follows:

1. Clean the glass slide or cover glass very thoroughly.
2. Place a drop of sterile water on one end of the slide or in the middle of the cover glass.
3. Transfer with a sterile needle a little of the growth to the drop of water and gently mix it with the water. Spread.
4. Allow to dry in the air or by gently heating above the flame.
5. After drying, fix by passing through the flame. This will give the "film" or "mount" which may be subjected to any of the following staining procedures:

[4] Details for the preparation of most of the common staining solutions are given in the Appendix.

General Stains for Microbiology

Bacteriologists have not developed the wide list of stains that biologists have. Staining solutions have not been altered much from those formulæ which were proposed by the early investigators.

Aqueous Alcoholic Stains.—These staining solutions are prepared by diluting a saturated alcoholic solution of the dye with distilled water. Many different aqueous-alcoholic solutions of dyes are used. The following procedure is used for any of them.

1. Flood the film with the staining fluid (methylene blue, carbol fuchsin, etc.). A little experience will indicate just how long the stain should be left on the film.
2. After about a minute, wash the staining fluid off with running tap water.
3. Dry and examine.
4. If the stain has been made on a cover glass this should be mounted on the end of a slide by means of Canada balsam if a permanent mount is desired. When the film has been made on the end of a glass slide, no cover glass is necessary. The immersion oil may be put directly on the stained film.[5]

THE GRAM STAIN

In 1884 Gram proposed a method of staining bacteria which has been used for distinguishing bacterial species from one another. Many different procedures or modifications of Gram's original method, and numerous explanations of it, have been proposed at various times. Bacteria are said to be either Gram-positive or Gram-negative, depending upon whether they retain the gentian violet or not. Those species which retain the violet color after staining with Gram's method are said to be Gram-positive; those which lose the violet color and assume the color of whatever stain is used as the counterstain, are said to be Gram-negative.

The explanation of this stain is not definitely settled. Gram in his original work did not attempt to explain it. Unna (1887) put the explanation on a chemical basis. The Gram stain is an arbitrary procedure, and variations in the details are very important. Some believe that the pararosaniline (methyl violet, gentian violet) compounds form substances with iodine which are insoluble in alcohol. The iodine salts of rosaniline (fuchsin and methylene

[5] For drying slides the author has found the bibulous paper booklet made by H. Reeve Angel & Co., Inc., 7 Spruce Street, New York City, very useful. Clean blotting paper may also be used.

blue), however, are decomposed by alcohol. The Gram-negative bacteria are stained only with the gentian violet. The alcohol washes out the iodine. The iodine in Gram-positive bacteria forms a compound between the protoplasm of the cell and the dye, which is insoluble in alcohol. In the Gram-negative bacteria this compound is soluble in the alcohol and is broken up. Kruse (1910) attempted to explain this stain by stating that the Gram-positive bacteria are more resistant to autolysis, solution in strong alkali, etc., than the Gram-negative species. Eisenberg (1909) assumed that the differential Gram staining rested upon the presence of some special compound, such as unsaturated fatty acids or phosphatids, in the cell membrane, and that these formed a compound with iodine which rendered the cell wall impermeable to alcohol.

Dreter, Scott, and Walker (1912), in seeking an explanation for this stain, proposed the presence of lipoidal substances in the Gram-positive bacteria which bind the pararosaniline compounds. This, however, has little foundation according to our present knowledge. Brudny (1908) regarded differences in the permeability of the cell membrane as the important factor. With the Gram-positive bacteria the membrane is more easily penetrated by the iodine solution. The structure of these bacteria is probably "looser" and the stain gains an easy entrance. The Gram-negative bacteria, however, are not penetrated, and no deposit of the dye takes place on the interior of the cell. Benians (1912) bases the explanation of this stain on a definite cell membrane which prevents the removal of the dye by the alcohol, while Hottinger (1916) explains it from the standpoint of colloidal chemistry. He states that the relation of the bacteria to this stain depends upon the degree of dispersion of the nucleoproteins. In the Gram-negative bacteria, the stained nucleo-proteins are so widely dispersed that they are not seen. In the Gram-positive bacteria the stained nucleoproteins form a coarse emulsoid. Gram-positive bacteria are said to become Gram-negative when the degree of disperson is increased by the action of proteolytic enzymes. Gram-positive bacteria resist ferment action longer than Gram-negative ones. Weinkopff (1911), in studying tryptic digestion of bacteria, concluded that the difference between the Gram-positive and the Gram-negative bacteria rested in the penetrability of cell protoplasm. Dreter *et al.* (1912) extracted a substance from Gram-positive bacteria (staphylococci) which, when applied to Gram-negative *B. coli*,[6] made them Gram-positive in many cases. When this ether extract was dried on a slide, the residue took the Gram stain. This investigator also treated *B. coli* with lecithin, which made them Gram-positive. That the Gram-negative bacteria are structurally different from the Gram-positive ones seems to be indicated by the work of Larson, Hartzell and Diehl (1918). These investigators stated that the Gram-negative bacteria could be broken up by the sudden release of the pressure when they were in an atmosphere of carbon dioxide, while the Gram-positive species resisted such treatment. The latter, although killed, underwent no morphological

[6] The name of this microórganism according to the newer classification is *Escherichia coli*. In most cases the author has tried to use the newer names with the old ones in parentheses.

change. A most comprehensive study and analysis of the Gram stain has come from the pens of Hucker and Conn of the New York Agricultural Experiment Station, Geneva, New York. Nineteen of the different technics were subjected to careful comparative studies.

Benians (1920) has continued his work to explain why organisms retain the Gram stain. The Gram-positiveness of certain bacteria rests on the physical structure of the cell contents. The mordant seems to have no relation to it. In Gram-positive bacteria there is formed a large molecular complex which does not pass out through the cell membrane in alcoholic solution. It has been reported (Churchman, 1921) that in a single bacterial culture there may be two types of individuals which may agree in many characteristics but may differ in their reaction to gentian violet. One will grow vigorously while the other will not grow at all. The factor that determines the action of an organism toward the Gram stain is not necessarily the one that governs its cultural behavior toward gentian violet. Consequently, Churchman believes that the chemical-affinity hypothesis must be given up as the sole explanation of the parallelism between Gram and gentian violet reactions. Benians recognized three groups of bacteria in relation to Gram staining, as follows: 1, Gram-positive bacteria into which the dye penetrates but which resist decolorization; 2, Gram-negative bacteria, like the gonococcus, into which the dye penetrates but from which the dye-iodine compound is readily washed out; 3, Gram-negative bacteria, such as the members of the *coli* group, into which the dye does not readily penetrate and is not absorbed into the cell and which are readily decolorized. Dargallo (1921) observed a difference in Gram staining ability which varied with the age of the bacteria. Old bacterial cells which tended to be Gram-positive when young often became Gram-negative in the later stages of development.

Friedrich (1920) has stated that the Gram-negative organisms possess a high resistance to the halogens, while the reverse is the case with the Gram-positive ones. The same observation has been reported for iodine by Breinl (1920). Gram-negative bacteria were found to be three times as resistant to iodine as the Gram-positive species.

Deussen (1921) studied the reasons for Gram-positiveness of bacteria and reached the conclusion that some substance is contained in the cell substance which has a special affinity for the dyes used in the Gram stain. This substance was said to be removed by acids or bases. When acids were used the effect was determined by the degree of dissociation, temperature and concentration. The author stated that the correctness of his hypothesis could be proven if he could remove the Gram-positive substance from cells, thus making them Gram-negative, and restore the Gram-positiveness by the addition of this Gram-positive substance. He continued his work with baker's yeast, which he treated with from 2 to 4 per cent caustic soda solution; this solution was then treated with hydrochloric acid and centrifuged. The yeast thus obtained was quite Gram-negative but still retained particles which fixed the Gram-stain reagents. When such yeast was treated with a mixture of nuclein and nucleic acid the cells became Gram-positive again. Yoghurt bacilli which had been rendered Gram-negative were made Gram-positive again by treatment with nuclein-nucleic acid solutions. Deussen believed that the

cell membrane interfered with the decolorization of the cell by alcohol. He stated that the nucleic acid and nucleins were diffused through the cell only in their sodium combinations and that the aqueous solutions did not penetrate the cell wall.

A possible source of error in the Gram procedure of staining has been mentioned by Sheppe and Constable (1923). They found that Lugol's iodine solution, which is neutral when made up, might undergo changes resulting in the formation of acid; this acid was said to be capable of decolorizing Gram-positive bacteria. Sheppe and Constable suggested that the good results obtained by Burke after the addition of alkali to the gentian violet might result from a neutralization of acids. To prevent this decomposition, it was stated that the iodine solution should be stored in brown glass bottles in a cool place and that the acidity could be neutralized by sodium bicarbonate. This seems to be borne out by Kilduffe (1923).

Hucker's Modification of the Gram Stain.—The following solutions are needed.

Solution A

Crystal violet (85 per cent dye content)	4	grams
Ethyl alcohol (95 per cent)	20	c.c.

Solution B

Ammonium oxalate	0.8	gram
Water	80.0	c.c.

Mix solutions *A* and *B*.

Lugol's Iodine Solution

Iodine	1	gram
Potassium iodide	2	grams
Water	200	c.c.

Counterstain

Safranin (saturated solution in 95 per cent alcohol)	10	c.c.
Water	100	c.c.

Procedure: Stain for one minute with the crystal violet solution; wash in water and then stain for one minute with the iodine solution; wash in water and dry with bibulous paper. Decolorize in 95 per cent alcohol for thirty seconds; counterstain for ten seconds. Wash, dry and examine.

Burke's Modification of the Gram Stain.—The following method was proposed by Burke (1922) and was said to yield better results than any of the other known methods.

1. Air-dry a thinly spread film and fix with the least amount of heat necessary to kill the organisms and fix them to the slide (*A*).
2. Flood smear with a 1 per cent aqueous solution of the dye to be used.

Mix with the dye on the slide 3 to 8 drops of a 5 per cent solution of sodium bicarbonate, allow to stand two to three minutes (*B*).

3. Flush off the excess stain with the iodine solution,[7] cover with fresh iodine solution and let stand one minute or longer (*C*).

4. Wash in water as long as described, and blot off all free water until surface of film is practically free of water, but do not allow the film to become dry (*D*).

5. Decolorize with acetone or acetone and ether (1 part ether to 1 to 3 parts acetone) until decolorizer flows from slide practically uncolored. This usually requires less than ten seconds (*E*).

6. Blot dry. The slide quickly dries without blotting (*F*).

7. Counterstain for five to ten seconds, or longer if desired, with a 2 per cent aqueous solution of Safranin O (*G*).

8. Wash off excess stain by short exposure to water, blot and dry (*H*).

Immerse in xylol or turpentine for several minutes or until clear. Examine.

If the first attempt at staining a smear does not give satisfactory results, it is advisable to wash off the oil with xylol, wash off the xylol with acetone and restain. It has been the author's experience that restaining films gives better results than the original attempt.

A. The film can be made in either distilled or tap water or in physiological salt solution.

B. Some workers recommend cooling the slide before flooding with the dye. With some dyes steaming seems to improve the result or shorten the required period of exposure. Passing the slide through the flame until steaming begins and allowing to stand the two minutes is sufficient. Steaming does not cause the Gram-negative organism to resist decolorization. Anilin-gentian-violet can be used in place of the aqueous solution if desired. Allowing the stain to dry around the edge makes a dirty slide but does not affect the Gram reaction. The strength of the solution of the dye and the period of exposure can vary somewhat without affecting the result. A saturated instead of a 5 per cent solution of sodium bicarbonate may be used.

C. The excess stain can be blotted off or washed off by a brief exposure to water. The exposure to the water should be as brief as possible, as water tends to reduce the amount of dye in the cells. Washing with water has the advantage of giving cleaner slides and effecting a saving of the iodine solution and the decolorizer.

D. The iodine solution can be blotted from the slide, but this has the disadvantage of leaving a small amount of iodine on the slide, and with the volatilization following the addition of acetone there is some irritation of the exposed mucous membrane of the worker. If for any reason one cannot complete the staining process after reaching this stage, it is advisable to place the slide in water until the staining can be completed.

E. The decolorizer should be placed upon the slide, allowed to stand for a few seconds and drained off. Then fresh decolorizer should be allowed to flow over the surface of the slide until it drops off clear. Proper control of this process will reduce the amount of decolorizer used to a minimum. Placing

[7] One gram iodine, 2 grams potassium iodide, 100 c.c. distilled water.

the decolorizer in a Coplin jar and dipping the slide up and down in it will not give satisfactory results.

F. Drying after the decolorizer is essential, as the aqueous counterstain, mixing with the decolorizer, has an effect on the Gram-positive organisms. The film can now be examined for Gram-positive organisms. The oil can then be washed off with xylene, the slide dried and the counterstain applied.

G. Various counterstains can be used but the author prefers Safranin O, or neutral red as recommended by Jensen. With dyes giving a poor Gram-reaction, it is necessary to reduce the counterstaining to a minimum.

H. Washing should be sufficient to remove the dye from the surface of the organisms; flushing for a few seconds will suffice.

Reaction of Some Common Bacteria to the Gram Stain.—

Gram-positive	Gram-negative
Staphylococcus citreus	*Neisseria intracellularis*
Streptococcus pyogenes	*Neisseria gonorrheae*
Streptococcus fecalis	*Vibrio comma*
Streptococcus lactis	*Vibrio proteus*
Gaffkya tetragena	*Escherichia coli* (*Bacterium coli*)
Staphylococcus aureus	*Aerobacter aerogenes* (*Bacterium aerogenes*)
Micrococcus cereus	*Eberthella typhi* (*Bacterium typhosum*)
Micrococcus aurantiacus	*Alcaligines abortus* (*Bacillus abortus*)
Spirillum rubrum	*Alcaligines melitensis* (*Bacillus melitensis*)
Bacillus simplex	*Alcaligines fecalis*

Gram-positiveness and Gram-negativeness are not always constant characteristics. For instance, *Lactobacillus bulgaricus* often has Gram-positive cells in the young cultures and Gram-negative cells in the old ones. Examination of the various steps in the procedures for the Gram stain also reveals the fact that this stain depends on arbitrarily selected steps.

Hucker and Conn carried out a very useful study of a number of methods of making the Gram stain.[8] Their conclusions are pertinent enough to be reproduced here.

"After a general survey of nineteen different methods of Gram staining, it is very difficult to select any one method as superior to all the others. The four methods denoted in Table I * as Jordan (1908), Buchanan (1911), Atkins (1920), and Hucker (1921) seemed, in the present investigation, to give the most satisfactory results, and they are probably all equally efficient when fresh mixtures of the stain are used and the time of decolorization is kept under two minutes. In general laboratory use, however, where directions for the

[8] Hucker, C. J., and Conn, H. J., Methods of Gram Staining. New York Agr. Exp. Sta., Tech. Bull., No. 93, 1923.

* This table will not be reproduced here. It may be consulted in the original bulletin.

time of staining and decolorizing are often loosely interpreted and where it is not always practical to make up fresh solutions every time cultures are stained, all of these four methods are not equally satisfactory in every case. Two or them, namely, the methods of Atkins and Hucker, use for mordants anilin sulfate and ammonium oxalate, respectively, neither of which has any harmful effect on the keeping qualities of the staining solutions, while both are such efficient mordants that they allow ample latitude in the time of decolorization, hence, these two methods are regarded as especially useful.

"A comparison of the various strengths of alcohol shows that little difference can be found between the results with 95 per cent or absolute alcohol, but that these two strengths give much more constant results than with alcohol containing more water. For this reason it is important, as pointed out by Burke (1922), that slides be carefully drained and blotted before putting on the alcohol, so as to prevent diluting it.

"Safranin, pyronin, Bismarck brown, and eosin were found more satisfactory as counterstains than fuchsin at the strength used. Of them, pyronin and Bismarck brown gave the most constant results, but safranin was not much inferior in this respect and is often quite desirable on account of the sharp contrast it gives with the color of Gram-positive organisms.

"The authors feel that the Gram stain is a variable reaction even under the most carefully controlled conditions and no worker should base his results upon a single observation. It is recommended that, in order to determine the tendency of an organism with regard to the Gram stain, preparations of the culture be made at various stages of growth, from twelve hours to several days in age. All preparations should be made in triplicate on separate slides. In this manner a broader conception of the staining reactions of a culture may be secured than by the usual procedure. It is advisable, also, if possible, to stain the organism by more than one method in order to eliminate the possibility of a faulty technic.

"Burke (1922) points out that the Committee on Bacteriological Technic should select carefully two cultures, one as a Gram-positive, the other as a Gram-negative strain, taking care to choose for this purpose two organisms that lie close to the border line between these two groups. Burke claims that in this way better standardization of the Gram stain can be obtained than by trying to standardize the technic itself. This statement is undoubtedly true, and it is not impossible that two of the four cultures used in the work listed in Table II * could be used for this purpose. Both the selection and distribution of such cultures will offer great difficulties; but it is, nevertheless, a matter to be given careful consideration.

"Whatever is done in the way of standardizing the Gram stain, it must be definitely recognized that not all organisms are distinctly Gram-positive or Gram-negative; and that a large number should be placed in a class to be regarded as Gram-variable, although a tendency one way or the other may be noted and recorded."

* See foot-note on page 91.

EXERCISE NO. 2: GRAM STAINING OF A MIXTURE OF TWO MICROORGANISMS

1. Prepare a mixture of two bacteria, one Gram-positive and the other Gram-negative. *Escherichia coli* and *Bacillus subtilis* are suggested. This mixture may be prepared by inoculating 1 or 2 c.c. of sterile water with a loopful of these organisms.

2. Make a film on a clean glass slide. Fix.

3. Stain according to Gram's method. The value of this differential staining procedure is well revealed in this manner.

THE SPORE STAIN

The spore is a resistant unit in the cells of some microorganisms. It shows the same resistance to the penetration of the dyes used in staining that it does to other unfavorable agents. This fact is used for determining the presence or absence of spores in bacterial cells. The ordinary staining procedures which have been outlined above give information on the presence of spores. The spore stands out as a colorless or very faintly stained granule in the cell. The vegetative protoplasm about the spore takes the stain. In the special staining procedures an attempt is made to stain the spore, if it is present, one color, and the vegetative protoplasm another. On account of the structure of the spore and its ability to resist unfavorable conditions, these methods are quite different from the ordinary staining procedures.

Preparation of the Film.—The same method is used for preparing the film for the spore stain that is used for any of the other methods of staining. In general, it is, perhaps, better to use an old culture since old cultures more often contain spores.

Neisser's Method for Staining Spores.—Flood the film with aniline fuchsin and keep it hot (steaming) for about an hour. Wash in water and decolorize with acid alcohol (1 part of hydrochloric acid and 3 parts of alcohol). Too long decolorization may remove the stain from the spores; consequently this step should be watched. Counterstain with methylene blue if desired.

The spores are red and the vegetative protoplasm blue.

Chromic Acid Method for Staining Spores.—Place the film in 20 per cent aqueous chromic acid for five minutes. Wash in water and stain with carbol fuchsin for thirty minutes. Wash, and examine under the microscope.

May's Spore-Staining Procedure.—This method was suggested by May as especially suitable for students. The procedure was outlined as follows:

1. Make a film and fix in the usual manner.
2. Cover with a small amount of 5 per cent chromic acid.
3. After thirty seconds add about twice as much concentrated ammonia as there is chromic acid on the slide. Allow to act about two minutes.

4. Rinse with tap water.
5. Steam with carbol fuchsin for two or three minutes.
6. Rinse.
7. Destain with 1 per cent sulfuric acid for from fifteen to thirty seconds.
8. Rinse again and flood the slide with tap water.
9. Add to this a few drops of Loeffler's methylene blue and allow to stain for from ten to thirty seconds.
10. Rinse, blot, dry and examine.

CAPSULE STAINS

The capsule is a gelatinous coating about the cell. Its properties require the use of special staining procedures for demonstrating its presence.

Muir's Method.

I. Mordant.

Mercuric chloride (sat. aq. sol.)	2 c.c.
Tannin (20 per cent aq. sol.)	2 c.c.
Potassium alum (sat. aq. sol.)	5 c.c.

II. Stain.

Carbol fuchsin.

III. Counterstain.

Methylene blue.

The film is mordanted two minutes, washed in alcohol and water and then stained two to three minutes with gentle heat. It is then washed with water and remordanted again for two or three minutes. Counterstain with methylene blue.

Muir's capsule staining procedure gives preparations in which the bacterial cells are stained a deep crimson and the capsules blue.

Welch's Method.—Fix the film in glacial acetic acid. After a few seconds pour off the acetic acid and flood with aniline gentian violet. Repeat until all acid is removed. Wash and examine.

The capsules will appear as a lavender-violet halo about the cell.

Huntoon's Method.—*Preparation of Reagents. Solution* 1.—To be used as diluent. Three grams of nutrose are sifted into 100 c.c. of distilled water and heated to 100° C. in the Arnold sterilizer for an hour. Add 5 c.c. of a 2 per cent phenol solution to act as a preservative. Decant into a test tube and allow to settle. Employ the supernatant liquid as the diluent. (Since the supernatant liquid tends to become thinner by constant precipitation of the nutrose, the solution should occasionally be reboiled.)

Solution 2. Fixing and Staining Solution

2 per cent aqueous phenol solution...	100	c.c.
Concentrated lactic acid	0.25 to 0.5	c.c.
1 per cent acetic acid solution.....	1	c.c.
Saturated fuchsin in alcohol.....	1	c.c.
Carbol fuchsin, old solution.....	1	c.c.

This staining solution must be kept tightly corked.

Technic of Staining.—1. Employ the solution (No. 1) as a diluent, emulsifying the bacteria in 1 or 2 loopfuls and then spreading in as thin a film as possible with the loop. The use of the edge of a slide in spreading the film is not to be recommended.

2. Allow to dry in the air.

3. Cover the film with the fixative and the staining solution (No. 2) and allow to act for from thirty to forty-five seconds.

4. Wash quickly in water, dry and examine.

Hiss's Method.—The film should be prepared in some body fluid, such as blood serum, dried and fixed as usual. It is stained with an aqueous alcoholic stain, either fuchsin or gentian violet (5 c.c. saturated alcoholic solution), for a very short time. The stain may be driven into the cells with gentle heating. The dye is washed off by means of 20 per cent copper sulfate solution, and the film dried and mounted.

For slime-forming bacteria Conn (1920) reports that rose bengal is to be recommended. It seems to have affinity for bacterial protoplasm but not for the slime contained in the capsule. It is also claimed that it brings out the internal structure of the cells.

The Gins Method of Demonstrating Capsules.—Hagan has called attention to the value of the Gins method for demonstration of capsules. The procedure, which involves staining of India ink films of bacteria, is as follows (after Hagan):

1. Dilute the ink with an equal amount of sterile water, or dilute and sterilize.

2. Place a drop of the ink near one end of a very clean slide and carefully mix in a loopful of the bacterial suspension.

3. Then spread the mixture across the slide with the edge of a second slide. A properly prepared specimen should be uniformly spread and of a grayish color rather than black. (Films that are too thick may loosen after fixation.)

4. After drying in the air, fix the film with heat or by dipping in methyl alcohol.

5. Stain with any ordinary bacteriological stain. Microscopic examination will reveal well-stained bacteria lying in lacunae in the film of ink. The margin of the capsule is sharply delineated by the ink and the margin of the cells by the stain.

ACID-FAST STAINING

Acid-fast bacteria are bacteria which, after being stained by a special process, are resistant to decolorization with acids. The staining reaction has been of great value in diagnosis of tuberculosis, for the etiologic agent in that disease is acid-fast. The following bacteria are acid-fast.

Mycobacterium tuberculosis (*Bacillus tuberculosis*)
Mycobacterium leprae (*Bacillus leprae*)
Mycobacterium smegmatis (*Bacillus smegmatis*)
Moeller's grass bacillus (*Bacillus phlei*).

Different reasons, such as a thick membrane about the cell or peculiar structure of the cell contents, have been given to explain the acid-fast staining procedure. The real reasons for acid-fastness are clouded with conflicting data. A few of the reasons which have been offered may be mentioned. Miller (1916) grew tubercle bacilli in sperm oil and attributed the variations in the staining properties with carbol fuchsin to the production of free oleic acid in the interior of the rod. This acid was believed to be formed by the spores or round granules in the cell. Bulloch (1904), when studying the acid-fast properties of bacteria, isolated a wax and other substances in the nature of fats from acid-fast bacteria. The other constituents of the cells were not acid-fast. Baumgarten (1911) thought that unsaturated fatty acids were present in the acid-fast bacteria and united with the dye. Benians (1912) claimed that the cell wall was the important factor in retaining the dye. He stated " for the present the evidence points to the existence of a waxy layer enclosing a protoplasmic and fatty cell substance and conferring on the organism the property of resisting the penetration of acid and alcohol." Aronson (1910) claimed that the peculiar staining properties of the tubercle bacillus are due to the fat content and not to any peculiar kind of protoplasm. This waxy substance may be extracted by trichlorethylen. Long recently discussed the problem and suggested that acid-fastness was a result of two factors, the presence of an acid-fast wax and its distribution in the cell. Long stated that the evidence which was available lent more support to the second factor. He believed that the disposition of the lipin-protein in the cell played a rôle in acid-fastness.

The original procedure of acid-fast staining is known as the Ziehl-Nielsen stain. There are many modifications of this procedure, the following being typical.

1. Prepare film in the usual manner.
2. Flood with steaming carbol fuchsin for five minutes. The slide or cover glass may be immersed in the staining fluid and the fluid heated.
3. Wash in water.
4. Decolorize with 25 per cent hydrochloric acid. This will remove the red color.
5. Counterstain with methylene blue for about one minute.
6. Wash, dry, mount, if desired, and examine.

Basic fuchsin	1 part
Absolute alcohol	10 parts
Carbolic acid (1 to 20)	100 parts

FLAGELLA STAINS

This has always been a difficult procedure for many bacteriologists. The difficulty may be due to lack of care in some of the preparatory procedures. Smith (1905) mentioned some of the common errors.

1. Oily or otherwise dirty cover glasses.
2. Unsuitable cultures.
3. Breaking off the flagella.
4. Uneven distribution of too many bacteria.
5. Imperfect mordanting.
6. Excessive mordanting.
7. Understaining.
8. Overstaining.
9. Precipitates on the cover glass during some stage of the process.

The greatest error probably lies in dirty slides and cover glasses. These should be cleaned in strong alkali in order to saponify any animal grease which may be on them. Otherwise, the film will "roll" and not spread evenly. The culture that is stained must be young and vigorous. A small amount of this growth should be transferred to a tube of sterile water and incubated at 37° C. After several hours, some of this suspension should be put on slides and made to dry by placing the slides in the 37° C. incubator. The film should then be fixed and stained by any of the many processes.

A number of methods have been devised for staining flagella, any one of which may be used with success. Each worker may have a method which gives him most satisfactory results; he may secure unsatisfactory results with methods regarded as satisfactory by other bacteriologists. The following steps will help to secure good results.

1. Select a number of clean glass slides. Boil them in cleaning solution on an electric hot plate for five or ten minutes. Rinse in distilled water until all of the cleaning solution has been removed.
2. After these slides have been dried, place on them, by means of a sterile pipette, a water suspension of the cells on which the flagella are to be stained. This water suspension or sterile physiological salt solution suspension, should be prepared a little while before by gently transferring cells from a twenty-four hour agar slant to the water. This should be put into the 37° C. incubator until the film is to be made.
3. By means of the pipette used to transfer the cells in the water suspension to the slide, carefully spread the water over one end of the slide or over the cover slip. Be careful not to stir or agitate the preparation needlessly.
4. Place in the 37° C. incubator to dry. The cells will be caught by the evaporation with their flagella spread out around them.
5. Stain according to any of the methods mentioned in the following paragraphs.

Loeffler's Flagella Stain.—The following solutions are used.

Loeffler's Mordant

Tannic acid, 20 per cent	10	c.c.
Ferrous sulfate (sat. aq.)	5	c.c.
Basic fuchsin (sat. in alcohol)	1	c.c.

Loeffler's Stain

Basic fuchsin (sat. in alcohol)	2.5	c.c.
Carbolic acid	20.0	c.c.

The tannic acid solution should be fresh. The other solutions may be older.

1. After preparation of the film, cover with the mordant.
2. Heat for two minutes in such a manner that steam arises from the preparation.
3. Wash with tap water.
4. Drain and blot excess water.
5. Cover with the stain and heat for two minutes as before.
6. Wash, dry and mount.

The author has secured the best results with Loeffler's method. A double boiler is prepared from two beakers, the inner one being

supported by pieces of cork. The staining solution should be put into the inner beaker and the temperature raised to about 65° C. or until the stain steams. The slides are immersed in this solution and stained as long as required.

Plimmer and Paine's Modification of the Casares-Gil Flagella Staining Procedure.—Plimmer and Paine have proposed the following modification of Casares-Gil's method (Jour. Path. and Bact. 24 (1921), 286–288).

The following ingredients are employed in making up the stain.

Tannic acid	10.0 grams
Aluminium chloride (hydrated)	18.8 grams
Zinc chloride	10.0 grams
Rosaniline hydrochloride	1.5 grams
Alcohol, 60 per cent	40.0 c.c.

Place the solids in a mortar, add 10 c.c. of the alcohol and mix thoroughly, taking care to smash up all the zinc chloride. Stir in the rest of the alcohol slowly. At this point the mass goes gradually into viscous solution of a deep-red color, and in this state appears to remain stable for several years. For use, dilute with water, 1 part stain to 4 of water; when nearly complete precipitation occurs, a small amount remaining in solution as a balanced colloid.

Method of Application.

1. The culture must be eighteen to twenty-four hours old and must be removed carefully. Slides should be cleaned with chromic acid, and all grease removed by roasting them over a Bunsen burner. When they have cooled to blood heat, a small drop of the bacterial suspension is placed at one end, the slide tilted and the drop allowed to run down, or it may be smeared gently with a strip of gutta-percha tissue. Quick drying of the film seems to be important, hence the warm slide. No fixing is required.

2. Apply the diluted mordant, allowing it to stand one minute after mixing with water. Filter on to the slide and allow to stand for one minute, when a slight surface bronzing should be visible. Wash rapidly under the tap.

3. Flood the film with cold carbol fuchsin for five minutes, wash, dry and examine in oil. If satisfactory, mount in balsam or euparal.

The advantages claimed for this method are as follows:

1. The ease with which successful preparations are obtained, making it a useful stain in the hands of inexperienced students.

2. It is possible to have the background absolutely free from stain.

3. The flagella are uniformly stained without the appearance of granular deposits, and do not appear to be unduly magnified.

4. The mordant is a one-solution stain, seems to keep indefinitely in a well-stoppered bottle and is always ready for use.

5. The materials are relatively cheap, as compared with other methods in which osmic acid is used as a fixative.

Miss Thatcher (Stain Technology, Vol. 2 (1926), 143) has stated that improved preparations are secured if the mordant is diluted with an equal amount of water.

Van Ermengem's Flagella Stain.—

I. Van Ermengem's Mordant.

Osmic acid	50 c.c.
Tannic acid (aqueous 10–25 per cent)	100

Four drops of glacial acetic acid should be added to the above.

II. Silver Bath. A 0.25 to 0.5 per cent solution of silver nitrate in distilled water.

III. Reducing Bath.

Gallic acid	5 c.c.
Tannin	3 c.c.
Sodium acetate fused	10 c.c.
Distilled water	350 c.c.

The film should be covered with the mordant for fifteen minutes at 50° C. The mordant is then washed off in distilled water and alcohol. The film is then placed in a small amount of the silver bath and gently heated. Without washing, it is then put into the reducing bath and agitated until the solution begins to blacken. It is removed, dried and mounted for examination. The apparatus used in this procedure must be absolutely free from animal grease. Kuntze (1902) has suggested some improvements in Van Ermengem's procedure for staining flagella.

STAINING OF GRANULES

Some bacteria show the presence of granules when stained according to methods for demonstrating these bodies in cells. Such a method is used for showing the presence of diphtheria bacilli among the other microorganisms on specimens from suspected throats. *Lactobacillus bulgaricus* (*Bacillus bulgaricus*) is a suitable non-pathogenic microorganism with which to demonstrate this method. Albert's stain is given below as a useful one for staining granules.

Solution 1

Toluidin blue	0.15 gm.
Methyl green	0.20 gm.
Acetic acid (Glacial)	1.00 c.c.
Alcohol (95 per cent)	2.00 c.c.
Water (distilled)	100.00 c.c.

After standing for one day, the solution is filtered and is ready for use.

Solution 2

Iodine	2 gm.
Potassium iodide	3 gm.
Water (distilled)	300 c.c.

The solution is ready for use as soon as the iodine is entirely dissolved.

Films are made on slides or cover glasses in the usual manner, fixed by heat and stained with the toluidin blue solution for five minutes. The stain is then drained off without washing and the iodine solution applied for one minute.[9] The slide is then briefly washed with water and dried, preferably by means of filter paper. It is now ready for examination.

Used Slides and Cover Slips.—It has been satisfactorily established that many of the ordinary staining solutions are not germicidal, and consequently slides and cover slips on which certain microorganisms have been stained should be regarded as dangerous. Slides and cover slips prepared with pathogenic microorganisms should be dropped into a 5 per cent phenol solution and left there for a week or so. This will insure destruction of the bacteria, after which the slides may be cleaned.

Preservation of Stained Preparations.—The microscopist or student may wish to make permanent preparations of some of the microorganisms with which he works. The details of such procedure may be found in books on bacteriological technic. Only the general procedures will be given here. After the preparation has been stained the slide should be dried and all dust and lint removed. Then a drop of Canada balsam or other mounting fluid should be applied on top of the film. A clean cover glass should be placed over the drop and carefully but firmly pressed down. The slide may be gently warmed to help soften the balsam. After the cover glass has been pressed down as far as possible, the edges should be cleaned with lens paper moistened with xylol, and the slide properly labeled and filed in a slide box. Such stained preparations may be kept for some time. The dyes tend to fade slowly, so that after a time they lose their original appearance.

[9] Albert, Henry, Diphtheria Bacillus Stain with a Description of a New One. Amer. Jour. Public Health (1920), 10; Modified formula published in J. Amer. Med. Assn., 76 (1921), 240.

Differential Counting of Living and Dead Bacteria (Henrici's Method).—This technic allows one to determine the relative proportion of living and dead bacterial cells in a suspension or culture and at the same time to secure information about the size and shape of the cells. Henrici described the technic as follows:

"A measured quantity of bacterial suspension is mixed thoroughly with an equal quantity of 2 per cent aqueous Congo red solution; the mixture is allowed to stand ten minutes. After again shaking the mixture, 0.01 c.c. is removed by means of a capillary pipette of that capacity and discharged on to a clean slide which has been clamped to the table over a piece of white paper on which a 2-cm. square has been ruled. By means of a stiff wire the drop of liquid is spread as evenly as possible over this area. After it has become thoroughly dry the slide is immersed a moment in a 1 per cent solution of hydrochloric acid in 95 per cent alcohol. This turns the dye blue and also fixes the film. If covered with a layer of cedar oil the slides will keep indefinitely, but if exposed to the air they fade considerably.

"Cells which were alive at the time of staining are unstained and appear as white spots on a blue ground. While the cells themselves may shrink considerably after fixation and drying, a number of comparative measurements have shown that the clear space in the film faithfully reproduces the size and form of the living wet cells. With favorable material, flagella may be demonstrated by this stain."

BIBLIOGRAPHY

BURKE, V. Notes on the Gram Stain with Description of a New Method. Jour. Bact. 7 (1922), 159–182.

CONN, H. J. An Investigation of American Stains. Jour. Bact. 7 (1922), 127–148.

ECKFELDT, G. A., and KOSER, S. A. The Germicidal Effect of Staining Solutions. Stain. Tech., 2 (1927), 109–112 (Good review of literature).

EYRE, J. W. H. Bacteriological Technique. W. B. Saunders Co.

HAGAN, W. A. The Gins Method of Demonstrating Capsules of Bacteria. Science 66 (1927), 173.

HUCKER, G. J., and CONN, H. J. Further Studies on the Methods of Gram Staining. New York Agr. Exp. Sta. (Geneva) Tech. Bull. 128, 1927.

MALLORY and WRIGHT. Pathological Technique. W. B. Saunders Co.

MOORE, V. A. Principles of Microbiology. A Treatise on Bacteria, Fungi and Protozoa Pathogenic for Domesticated Animals. The Macmillan Co., New York, 1916. 1st Edition.

STITT, E. R. Practical Bacteriology, Blood Work and Parasitology. P. Blakiston's Son & Co.

TANNER, F. W. The Bacteriology and Mycology of Foods. John Wiley & Sons, Inc., New York, 1919. Chapter III.

CHAPTER V

ISOLATION OF BACTERIA

By means of the cultural examination of microorganisms, their physiological, or functional, properties are determined. The microorganism is grown under controlled conditions in sterile media and the changes which it brings about are studied chemically or otherwise. The data from these examinations, along with those secured from morphological studies, are used to characterize and identify the organism. The microbiologist finds it necessary to use many different media and methods for studying bacteria. The characteristics revealed by them are known as differential characteristics.

Pure Cultures.—The first requirement for the study of an organism is its isolation in "pure culture." Pure cultures are just as necessary to the bacteriologist as "chemically pure" materials are to the chemist. The chemist could not secure reliable information if he did not have pure compounds. Neither could a bacteriologist determine the true characteristics of a microorganism if he did not have it in pure culture. Much of the pioneer work in bacteriology was probably carried out with impure cultures. The term *strain* is used for designating the growth of bacteria. It has a slightly different meaning from culture. A *culture* of a microorganism is its growth in or on a culture medium. By *strain* is meant the line or stock. A bacteriologist may have many strains of the same microorganism and he may also have many cultures of the same strain.

Dilutions.—It would be a tedious, if not an impossible, task to count bacteria in preparations which contained millions of cells per cubic centimeter or gram. In order to avoid this, these materials are diluted with sterile water. Usually a definite amount of the sample (1 c.c. or 1 gram) is added to water sterilized in bottles. They are often called "water blanks" and may contain any amount of sterile water. The amount, however, must be

known. Since the metric system is commonly used by scientists, the cubic centimeter and gram are used in bacteriology as the units of volume and mass. Bacteriological data are therefore reported per cubic centimeter or gram. Dilution blanks are usually made with 9 or 99 c.c. of water. Their preparation was discussed in Chapter I.

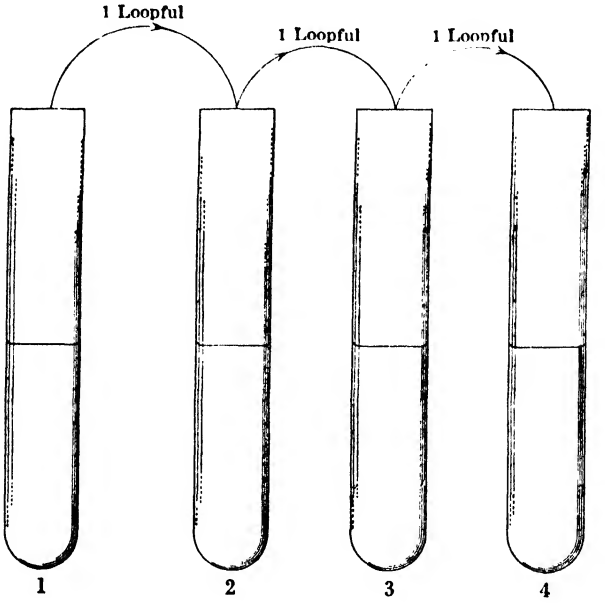


FIG. 39.—Method of Making Dilutions. The Tubes may Contain Sterile Water or Melted, Cooled Agar or Gelatin Medium.

For qualitative dilution transfer a loop of the sample (water, sewage, milk, or other material) to the first tube. After thorough shaking, transfer a loopful of this dilution to the second tube, and so on. If melted media have been used the contents of the tubes may be poured into sterile Petri dishes and allowed to harden. If sterile water has been used in the dilution tubes, 1 cc. or a loopful of the contents of each tube may be added to a tube of melted agar and this mixture poured into a sterile dish.

Qualitative Method of Dilution.—By this method the sample is diluted without giving thought to the exact extent of dilution. Dilutions may be made in any sterile medium, such as distilled water, plain broth, melted agar, or gelatin, etc. The greatest care must be exercised, however, to prevent the ingress of organisms from the air, etc. The object of diluting samples is to suspend the bacteria in a larger unit-volume of sterile liquid. When an

aliquot portion of this is taken for further study, a smaller number of bacteria will thus be taken. The unit of volume transferred may be expressed in drops, loopfuls or any other unit. Let us suppose that we wish to isolate the organisms in sewage into pure cultures for more intensive study. Sewage may have 3,500,000 bacteria per cubic centimeter. If we wish merely to isolate the bacteria and do not care to enumerate the organisms (see following paragraph) we could transfer any unit volume, as follows:

1. Carefully shake the sample to be sure that the organisms are evenly distributed.
2. By means of a sterile loop transfer a loopful of this sample to a tube of sterile water.
3. After shaking this tube well to distribute the bacteria, transfer a loopful of this suspension to a second tube of sterile water and shake to distribute the organisms.
4. Repeat this if the sample is known or believed to have many microorganisms.
5. The final tube should have the same general types of bacteria in the same relative proportions as the original sample. Their concentration is much less. This tube may then be used for the preparation of Petri dish cultures as described elsewhere.

Dilution in Liquefiable Solid Media.—This is the method which is commonly used.

1. Shake the sample to secure even distribution of microorganisms.
2. Melt several tubes of plain agar (or gelatin, etc.) and cool to about 44° C. to 50° C.
3. Sterilize the loop in the flame, and, after it has cooled, transfer a loopful of the sample (sewage, milk, etc.) to the first agar tube and shake.
4. Repeat this for the second tube and continue for three or four tubes if the sample is known to contain many bacteria.
5. The agar may be solidified in the tubes to make "shake" cultures or "Esmarch tubes" if colony formation is to be studied. If not, proceed.
6. Pour these agar tubes into sterile Petri dishes as mentioned on an earlier page.

It must be quite apparent, if liquefied solid media are used for dilutions, that not all of the bacteria will be poured out of the tube if the agar is to be poured into Petri dishes. Consequently, this method of dilution should not be employed for quantitative work where it is desired to know the number of bacteria present.

Quantitative Method.—This is essentially like the qualitative method except that definite units of volume are used. These are

necessary because quantitative enumerations of the organisms will be made. The technic is as follows:

1. Shake the sample thoroughly and transfer, with a sterile pipette, 1 c.c. to a water blank containing 99 c.c. This will give a dilution of 1 : 100. If 1 c.c. of this thoroughly mixed dilution is added to another 99-c.c. water blank the original sample will have been diluted 10,000 times. This procedure may be repeated any number of times, depending on the degree of dilution believed to be necessary. (Follow the instructor's directions.)

2. After the sample has been quantitatively diluted, 1 c.c. may be transferred to (*a*) the bottom of a sterile Petri dish, (*b*) a culture tube, or any other contained. If transferred to the bottom of a Petri dish a tube of melted, cooled agar (or gelatin) medium is poured into the dish and the dish carefully rotated to mix thoroughly the medium and diluted sample. Allow to harden.

3. Incubate the Petri-dish preparation and count the colonies which develop. The "count" must be multiplied by the dilution factor in order to find out the number of bacteria per cubic centimeter of original sample.

4. The Petri dish should be inverted during incubation to prevent spreading colonies. These may develop anyway on account of the inherent characteristics of the organism or a damp rainy period.

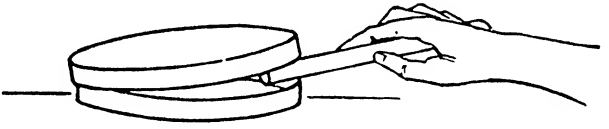


FIG. 40.—Showing the Method of Pouring Liquefied Media into a Culture Dish. (After Heinemann.)

Note that the cover is not removed entirely from the dish.

Preparation of Petri-dish Cultures.—With such preparations the microorganisms are separated from one another in liquefiable solid media. The bacteriologist calls this procedure *plating out*, and the preparations *plates*, or *Petri-dish preparations*. Each cell is held fast in the medium as the latter solidifies; it later develops into a colony of similar cells after the plates have been incubated under the proper conditions. The colony, then, represents the descendants of a single cell and is a pure colony if the sample has been properly prepared.

Such plates or cultures are prepared in pieces of apparatus called Petri dishes or bacteriological culture dishes. The form now used was devised by Petri, whose work is perpetuated in the name. The Petri dish consists of two glass dishes which fit into each other. This gives a cell or chamber in which the organisms

under study may grow without danger of extraneous forms entering.

1. Melt a sufficient number of tubes of sterile agar or gelatin or modifications of these media. The agar will require boiling while the gelatin will melt quickly at a temperature of about 50° C. or lower.

2. After the tubes of media are melted,[1] cool them to about 45° C. either in a water bath or in the air. For the qualitative dilution the tubes should be marked in 1, 2, 3, order.

3. Place the sterile Petri dishes on the table and make the necessary marks on the cover to identify the preparations after incubation.

4. Sterilize the needle or loop in the flame and, after allowing it to cool, transfer a bit of the specimen or sample (water, milk, sewage, soil, etc.) to the first tube of agar. After shaking, repeat the performance from the first to the second tube, and then from the second to the third. These inoculated tubes will then contain decreasing amounts of the sample and should be poured into the sterile Petri dishes. For quantitative work, sterile pipettes and known amounts of dilution water must be used.

5. For quantitative work place a unit volume (usually 1 c.c.) in the bottom of a sterile Petri dish and pour the melted cooled medium over it. Gently rotate the dish to distribute the sample evenly throughout the agar.

6. Great care should be used in pouring media from a test tube into a Petri dish. It is then that the medium and sterile Petri dishes are exposed to contamination.[2] The cover of the Petri dish should be raised very cautiously, just far enough to permit the entrance of the end of the test tube, and immediately replaced after the dish has received the liquefied medium. Thus, only the contents of the test tube are introduced. Flaming the mount of the tube before pouring the media, and care to avoid touching the inside of the sterile dish with the tube, will help to avoid contamination.

Method of Isolation of Pure Cultures.—There are different methods for doing this. The cover to the Petri dish should not be taken off until the cultures desired have been transferred to other culture media.

In order to be certain of pure cultures, the Petri-dish preparations must have certain characteristics. The colonies must not be too thick. They should be few enough to allow the bacteriologist to " pick " one colony without touching those close to it.

[1] Beginners often have trouble in making good plates or Petri-dish preparations because they do not completely melt the agar in the tubes. If the agar medium is incompletely melted, the plate preparation will contain lumps of the solid medium. If this is the case, plates should be poured again.

[2] When cultures are being transferred and plates poured, the technician should avoid speaking since it is easy to contaminate the cultures. There should be no air currents. Ideal conditions are difficult to secure in the student laboratory.

The colonies, if the Petri-dish preparation has been made from a pure culture, should show the presence of one type of organism. At least, one type of organism should predominate. The colonies should be carefully studied by means of a low-power hand magnifying glass in order to make certain that the one selected for transfer is not made up of more than one colony.

1. Examine the plates or Petri-dish cultures very thoroughly and record observations with regard to characteristics of the colonies. Numbers may be given to the colonies by writing on the bottom of the dish with a wax pencil.

2. Sterilize the needle by heating it to a dull red heat in the flame. Allow to cool.

3. Touch with the sterile needle a colony from which it is desired to prepare a pure culture, and transfer a little of the growth to a sterile agar slant (or other medium). Such colonies should, of course, be well isolated so that the

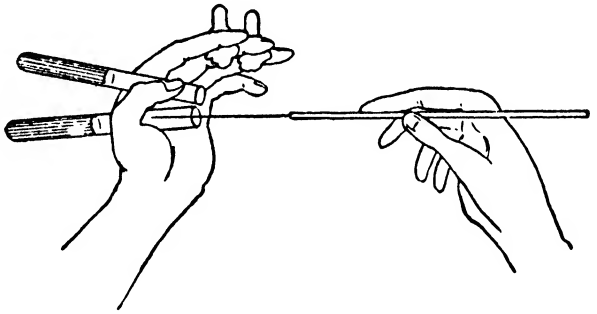


FIG. 41.—Showing one Method of Inoculating Media. (After Heinemann.)
Note the manner in which the plugs are held.

needle will not come in contact with another colony; this might give an impure culture. Remove the plug from the culture tube with the greatest possible care and insert the needle to the bottom of the slanted surface.

The agar slant should be streaked in a straight line from the bottom of the slant to the top. The inoculation should be on the surface. The needle should not be allowed to cut into the medium. Quite often there is considerable free liquid at the bottom of the slanted agar. This has been shown to be rich in nutrients and need not be thrown out. It will also help to keep the agar slant moist during its sojourn in the incubator. Agar slants with considerable moisture should not be laid flat on the desk, else the surface will become moist, causing the growth to spread.

In systematic studies the same colony should be used for the inoculation of all culture tubes. Often the colonies are so small that this may seem impossible. In order to accomplish this, the colony may be transferred to a sterile test tube containing a little

sterile water and thoroughly shaken. Loopfuls of this emulsion may then be transferred to the various culture tubes. In this manner a great number of culture tubes may be inoculated from a very small colony. A broth culture could also be made from the colony, and after a short incubation period loopfuls of this could be used for inoculation of culture media. This, however, would require more time.

Inoculation of Liquid Media.—Carefully twist the plug of the culture tube of liquid media without removing it, in order to loosen it from the tube. Sterilize the loop or needle by heating it to a *white* heat in the flame of the Bunsen burner. Also pass the lower part of the handle through the flame. Allow it to cool and, holding the needle like a pencil in the right hand, take the two culture tubes in the left hand. Remove the two plugs by grasping them with the back of the fingers of the right hand. The plugs must not be put on the desk, else they will become contaminated by organisms on it. The inoculation may then be made and the plugs replaced. The proficiency with which this is carried out will become greater as the laboratory work progresses. Avoid putting the handle of the needle into the medium while making the inoculation. This is one method by which the various things involved in the transfer of cultures may be manipulated.[3] Probably no one method will be satisfactory for all students. The student should understand the principles which are involved. It may be that he can develop his own technic of handling the tubes, etc., and not try to follow too closely any described method.

Preparation of Stab Cultures.—Such cultures are made with solid media such as agar or gelatin. They are employed to determine whether the microorganism is able to grow away from air or not.

1. Sterilize the needle in the flame of the Bunsen burner, allow it to cool and dip it into the culture from which the inoculation is to be made.

2. After carefully flaming the cotton plug, if necessary, remove it, and force the straight wire to the bottom of the tube. Withdraw the needle carefully.

3. Replace the plug, and incubate the culture.

4. Examine by holding the tube before a source of light and gently rotating.

[3] During the manipulations there should be no air currents to make dust nor should the manipulator talk, since it is known that bacteria may be expelled from the mouth when speaking.

Preparation of Shake Cultures.—The shake culture was an important step in the study of bacteria by the older methods. It is especially useful, in present-day methods, in the preservation of anaerobic pure cultures and their isolation from various materials; also for the study of colony structure. It may also be employed for demonstrating whether an organism is aerobic or anaerobic. If the organism grows down in the medium and refuses to grow near the top where oxygen may be absorbed, an anaerobic organism is indicated. On the other hand, if the organism grows only at the top of the culture, an aerobe is indicated. Shake cultures are prepared as follows:

1. Melt several tubes of plain agar or gelatin and allow to cool.
2. By means of a sterile pipette transfer a cubic centimeter or so of a water suspension of the organism or broth culture to the melted, cooled agar or gelatin. Push the pipette down into the medium to the bottom of the tube. Gradually withdraw the pipette as the inoculum flows from it so that the bacteria are distributed throughout the tube.
3. Allow to solidify and incubate.

Enumeration of Bacteria.—In sanitary work and other applications of bacteriology, it is often necessary to determine the number of bacteria in water, milk, sewage or other materials. The technic for this must be very carefully carried out and made to conform to standard procedures. Some of them have been discussed under another heading.

The methods for counting bacteria may be classified as follows:

A. Cultural Methods.
   1. Standard agar and gelatin plates.
   2. Frost's Little Plate Method.

B. Microscopic Methods.
   1. Breed's Microscopic Method.
   2. Haemocytometer.

Collection of Samples.—The success of the analysis is determined by the care with which the samples are collected and prepared. Careless collection of samples will often make laboratory analyses valueless. Obviously, since quantitative data are sought, it is necessary to exclude extraneous bacteria. Sterile bottles or containers must therefore be used for taking the speci-

men. After it (food, water, etc.) has been brought to the laboratory it must be prepared for analysis in a careful manner. This will consist of either grinding under aseptic conditions or shaking well before portions are removed for cultural work.

Standard Agar Plate.—A Petri-dish preparation is made in what may be called the standard agar plate. Since the data may be used for comparison, the plates should be made with standard materials and held under standard conditions.

The sample (or dilutions of the sample) should be thoroughly shaken and, if liquid, 1-c.c. portions placed in separate sterile Petri dishes. Melted agar or gelatin which has been cooled to 40°–45° C. should then be poured into the dish and mixed thoroughly with the sample by gently tilting and rotating the dish. The dish should then be placed on a level surface and allowed to harden, after which it should be incubated. The above procedure will suffice for samples that do not contain over 300 or 400, or less than 20 or 30, bacteria per cubic centimeter. Hill (1904) and Breed and Dotterer (1916) found that Petri dishes containing numbers of bacteria outside of these limits do not give accurate results. These Petri-dish cultures must then be incubated at 37° C. for twenty-four to forty-eight hours, after which the colonies are counted with the help of any of the various plate counters. The Jeffer plate counter is very convenient and is reproduced in Fig. 42. The results from plates made from dilutions must be multiplied by the required factor to bring them to a cubic-centimeter basis.

Often the plates may contain too many colonies, making it tedious or impossible to count the entire plate. If the colonies are evenly distributed, the numbers in several square centimeters may be counted, and the average number per square centimeter determined; this may be multiplied by the area of the Petri dish in square centimeters to determine the number of colonies on the whole dish. This is perhaps not as accurate a method as counting the whole plate but will suffice for ordinary work.

Different devices may be used for counting unit areas of Petri-dish cultures. A method sufficiently satisfactory under some conditions is to rule off the bottom of the dish by means of a wax pencil. In this manner one may keep from counting the same colony twice.

Streak Plates.—It is possible to prepare an agar plate culture by streaking the surface after the agar has hardened. This

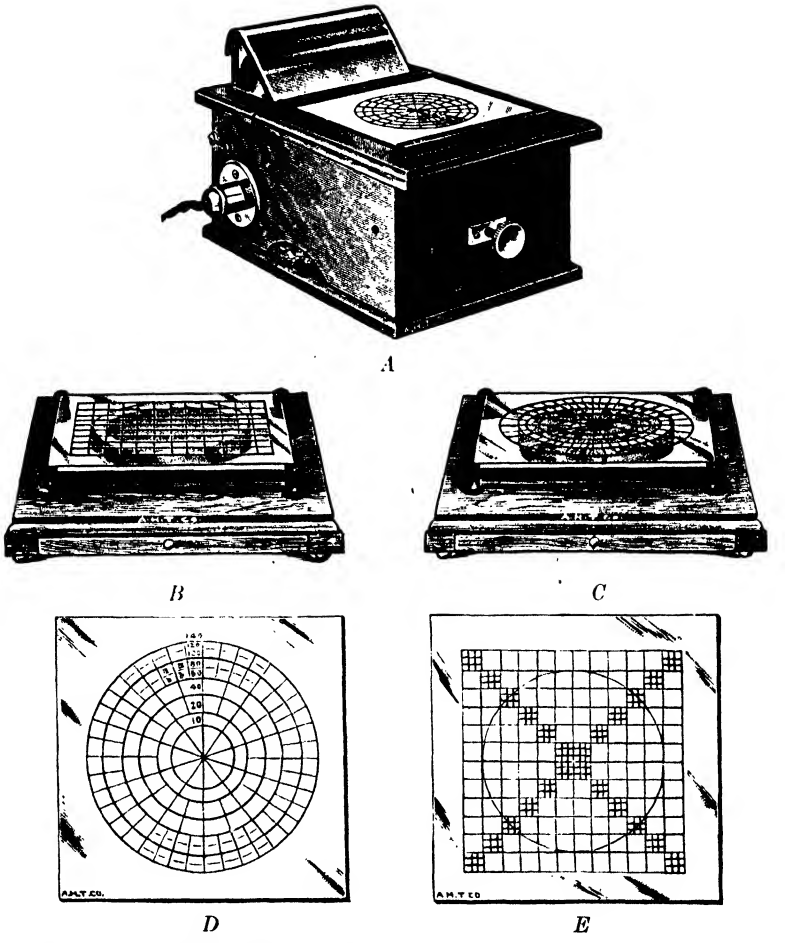


FIG. 42.—Showing Different Types of Apparatus for Counting Colonies of Microorganisms.

A, Steward's Counting Apparatus. *B*, Wolffhuegel's Counting Apparatus. *C*, Jeffer's Counting Apparatus. *D*, Counting Plate, Jeffer. *E*, Counting Plate, Wolffhuegel. (See Appendix for Frost Counting Plates.)

obviates the necessity of making dilutions but has the disadvantage that considerable care must be taken to get isolated colonies and that quantitative data may not be secured.

1. Liquefy the agar by boiling and cool to about 50° C.

2. Pour into the bottom of a sterile Petri dish and allow to become perfectly cold and hard.

3. Inoculate the loop with a bit of the sample (liquid or solid, broth culture, milk, feces, pus, etc.) and carefully raise the cover from the dish.

4. Starting at one side, carefully make streaks back and forth about ½ cm apart *on the surface* of the hardened medium. Each streak will leave a bit of the sample on the medium. *Do not reinoculate the loop or needle.* The object is to secure isolated colonies, and the streaks made at the last will probably give them after incubation. Some practice is necessary before satisfactory preparations will be secured by this method. Inexperienced individuals will find it advisable to continue the inoculation on another plate without reinoculating the needle. This gives greater certainty of securing isolated colonies.

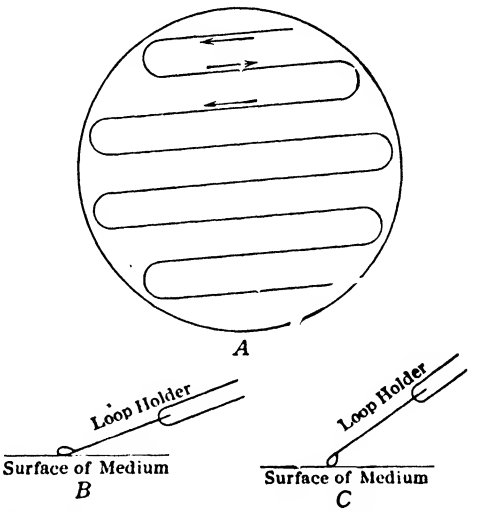


FIG. 43.—A Diagram to Show the Preparation of a Streak Plate.

A, A loopful of the specimen (feces emulsion, lactose broth fermentation tube, etc.), is carefully rubbed across the surface of the hardened medium back and forth without reinoculating the loop. *B*, Showing the manner of holding the loop for the first part of the plate. *C*, Showing the manner of holding the loop toward the end of the inoculation. This will allow the growth of isolated colonies. Students who are carrying out this procedure for the first time should continue the inoculation (without reinoculation of the loop) on another Petri dish containing hardened medium. This will give more experience and greater assurance of securing isolated colonies.

The microscopic methods for counting bacteria will not be discussed in this book. They may be found, if desired, in the author's "Bacteriology and Mycology of Foods."

Disposal of Cultures.—In the first course in general bacteriology, it is unnecessary to use pathogenic microorganisms. Some of the semi-pathogenic types, however, may be employed. All used cultures and apparatus resulting from the study of these types should be placed in a specially designated place for sterilization before washing. It is better, also, to have pans, etc., for holding such materials. These, with their contents, may then be placed in the autoclav for sterilization. What has been said of

pathogenic bacteria may also be said of thermophilic bacteria. Microorganisms that have a high temperature optimum also have a high thermal death point. All old culture materials from studies on thermophilic bacteria should be sterilized in the autoclav. If this is not done and the laboratory becomes infected, it is more difficult to be certain of sterile media.

ANAEROBIC METHODS

Many bacteria are not able to live in an atmosphere of oyxgen, and consequently if they are to be grown under artificial conditions attempts must be made to reduce the oxygen concentration. To accomplish this, many methods have been devised, some of which are quite complicated. Some involve the absorption of the oxygen by means of pyrogallol and sodium hydroxide. Others involve replacement of the oxygen by means of an inert gas such as nitrogen or hydrogen. The following methods are sufficient for introductory work.

Fermentation Tube.—One simple method for determining the ability of an organism to grow anaerobically is to find out whether it will grow in the closed arm of the fermentation tube. If it does, the ability to grow away from atmospheric oxygen is probably indicated. In order to make the observation more trustworthy, the dissolved oxygen should be removed from the tube by boiling before inoculation is made.

FIG. 44.—Buchner's Anaerobic Apparatus. (*Central Scientific Co.*)

Buchner's Method.—The culture is made on any medium desired in the ordinary laboratory culture tube. This may be either a streak on liquefiable solid media or a liquid culture. The culture tube is placed in a larger test tube or bottle which may be tightly stoppered. Into the bottom of this larger test tube are put about 3 or 4 grams of pyrogallol and about 10 c.c. of 10 per cent sodium hydroxide. After these have been mixed and the culture tube put in place, tightly stopper the bottle or tube and incubate. This mixture of pyrogallol and sodium hydroxide will absorb practically all of the oxygen and thus leave an atmosphere which will permit the development of anaerobic bacteria.

Wright's Method.—This depends upon the same principle involved in Buchner's method. Instead of using two test tubes, the culture tube itself is also used for the absorption tube. After the culture has been made, a tight plug of cotton is forced down to within about 1 cm. of the surface of the medium. On this plug are placed about 2 grams of pyrogallol and a little sodium hydroxide solution. The tube is quickly capped and placed in the incubator. Care should be taken to prevent the use of too much alkali. If too much is used it may run through the plug and enter the culture medium.

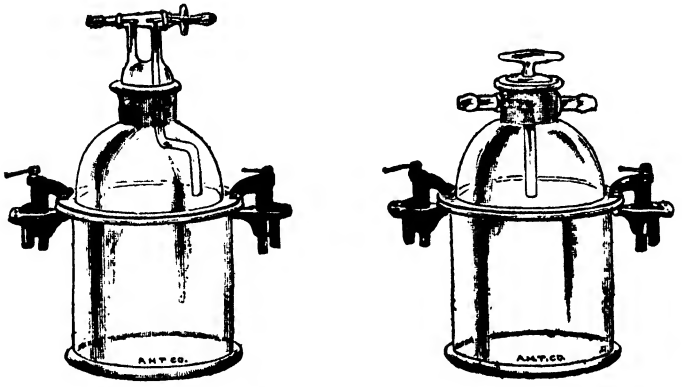


FIG. 45.—Showing Two Types of Novy Jars.

The tops on these jars are sealed with vaseline or stop-cock grease and clamped as indicated.

Replacement of Oxygen by Hydrogen.—Novy has devised a special jar for this, but any piece of apparatus that will allow the entrance of the hydrogen and the exit of air may be used. An ordinary wide-mouth bottle with two glass stopcocks will give as good results as the more expensive apparatus. The culture should be put into the jar and the rubber stopper carrying the glass stopcocks inserted. One of the glass stopcocks should be connected to the supply of hydrogen and hydrogen passed in until the gas escaping at the other glass stopcock will give the hydrogen pop.

Dick's Method.—Pour the inoculated agar into the Petri dish and allow to harden or pour the melted agar into the dish containing the sample. After it has hardened, pour a tube of melted agar over this and allow it to harden. Cover with melted paraffin

and incubate. Northrup has proposed the addition of a dye to the paraffin to make the colonies more easily visible. A fat-soluble dye, such as Sudan III, must be used. This is a useful method in that no special expensive apparatus is required. It has, however, the disadvantage that the paraffin is often hard to remove entirely from the glass.

Methods Using Ordinary Petri Dishes.—Many methods for growing bacteria anaerobically require the use of special apparatus. Two methods are given here which require only the use of ordinary Petri dishes.

Torrey's Method.—This method consists of growing an aerobic and an anaerobic organism together. A sterile Petri dish is

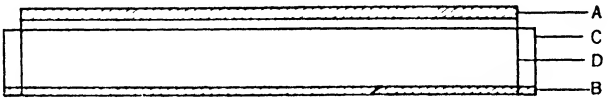


FIG. 46.—Cross Section of Torrey's Anaerobic Plate.

A, Solid medium containing the sample or organism to be grown anaerobically; *B*, Agar heavily inoculated with an aerobe (*B*, subtilis, etc.); *C*, and *D*, Petri dish halves which form the chamber.

poured and inoculated in the usual manner with the medium and the anaerobic organisms or sample to be used. The dish is then placed in the incubator for a short time to remove excess moisture and to dry the sides of the dish. After drying, the bottom of this dish is inverted into the cover of another dish into which agar inoculated with an aerobic bacterium has been poured. This makes a closed space in which the free oxygen will be used by the aerobic organism, creating anaerobic conditions for the anaerobe.

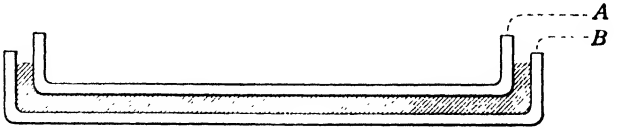


FIG. 47.—Showing method of securing anaerobic conditions with ordinary Petri dish.

A, Bottom of the dish; *B*, Top of the dish. Shaded portion the medium. This method is suitable for short incubation times. Prolonged incubation may result in evaporation of the medium.

Krumwiede and Pratt's Method.—This method is especially useful although it has the disadvantage of allowing a little evapora-

tion of the medium during prolonged incubation periods unless sterile melted paraffin is used. The apparatus required consists of the two halves of a Petri dish, sterilized with the bottom resting in the cover as shown in Fig. 47. The inoculated medium is poured into that half which is usually the cover, and the other half, usually the bottom, is placed in the liquefied medium.

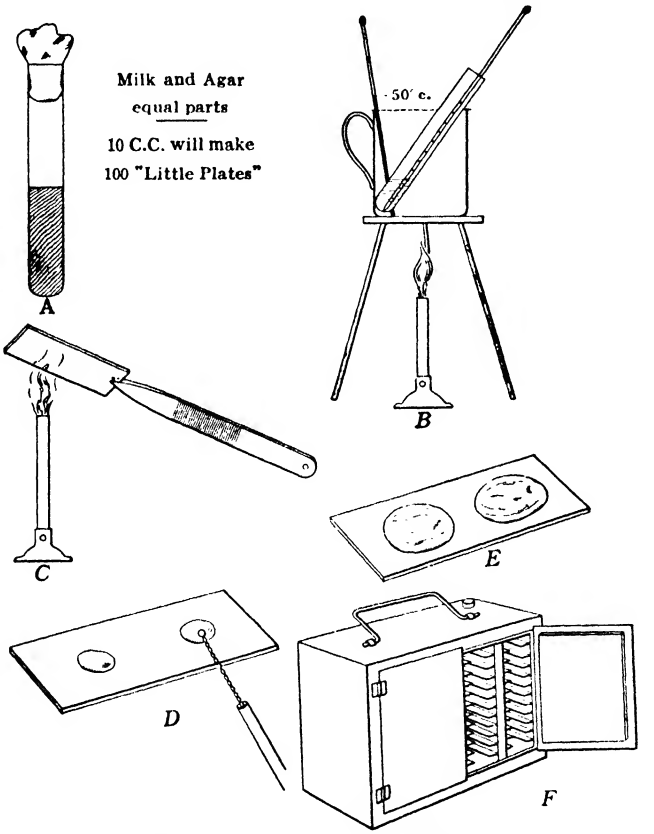


FIG. 48.—Method of Making "Little Plates." (After Frost, 1916.)

A, milk and nutrient agar, sterilized separately and mixed in equal quantities; *B*, medium, melted and ready for use; *C*, flaming the slide; *D*, inoculating the medium and spreading the drops; *E*, slide ready for incubation in the moist chamber, *F*.

Frost's Method for the Study of Bacteria.—Frost (1919), who devised a microscopic method for determining the number of bacteria in milk, has so altered that method that it may be used for the study of types of colonies of various bacteria. The colonies

are very young ones and must be examined with the low-power optical combination on the microscope. Frost described his technic as follows:

" The medium in its simplest form is a 50–50 mixture of nutrient agar and sterile milk.

" The apparatus is of such a simple nature that it will be found in all bacteriologic laboratories. It includes pipettes, microscope, glass slides, platinum needles, an incubator, common stains, etc. The pieces of apparatus that could be called special are a ' moist chamber cabinet,' a warm plate for

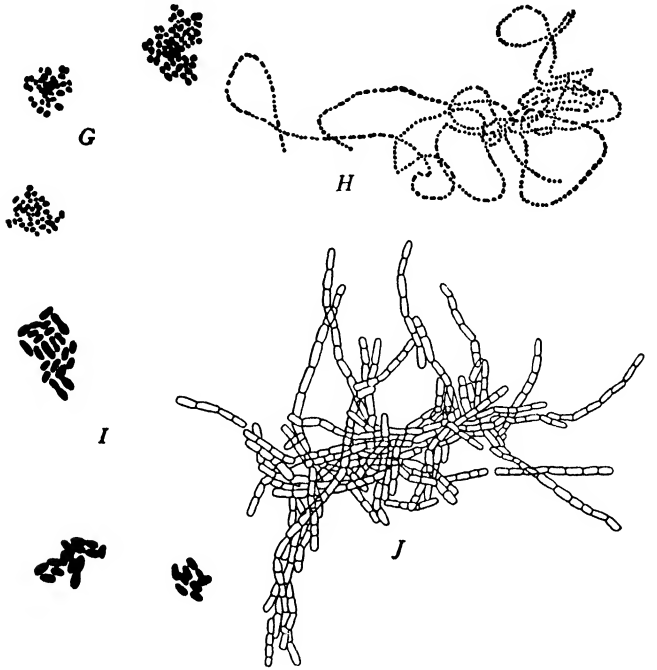


FIG. 49.—Colonies, Four Hours Old, of Organisms Grown on the "Little Plates." (After Frost.)

G, Micrococcus pyogenes var. aureus; *H*, Streptococcus pyogenes(?), from a throat culture; *I*, Bacillus coli; *J*, Bacillus subtilis. The drawings were made by Miss Margaret Brown under the camera lucida, and give the relative size as well as the shape and arrangement of the individuals in the colony.

drying the films, and forceps for handling microscope slides; but makeshifts for these are easily found in the common equipment of all laboratories.

METHOD OF MAKING CULTURES

"A clean glass slide is sterilized in the direct flame and placed on the table. When it has cooled down there is placed on it, from a pipette, two separate,

medium-sized drops of the culture medium, which has been previously melted and cooled to about 50° C. Into one of the drops of the culture medium, the material containing the bacteria to be cultivated is introduced by means of a platinum loop and thoroughly mixed, while at the same time the drop is spread over the slide in a thin film. When the first drop is spread, the loop is carried over to the second drop, which is thus inoculated with a small fraction of the same material. This drop is spread in the same way as the first. As soon as this is done, the slide is put in a moist chamber and placed in the incubator. In this way two little plates are made on the same slide from each sample to be examined, containing quite different amounts of the inoculum.

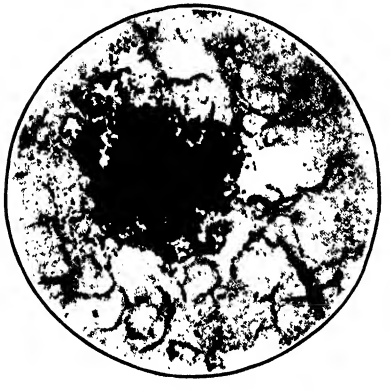


FIG. 50.—A Colony of Bacteria on the Frost Little Plate. (After Frost, 1916.)

A colony five hours old under the oil-immersion objective showing the individual cells and the arrangement during growth—probably *Escherichia acidi-lactici* (*Bacillus acidi-lactici*). The reticulated structure of the medium is due to the fat globules in the milk which was used with the agar. This also accounts for the open spaces in the colony.

"It is necessary to work rapidly in making these 'little plates' in order to prevent the agar from hardening before it is spread. It is also necessary to use aseptic technic at every step, except that I have not found it necessary to take any special precautions against air infection during the preparation of the plates, or to keep the edges and under side of the glass slides sterile. The method is illustrated in Fig. 48.

INCUBATION

"Immediately after being made, the little plates are put in a moist chamber where they are kept until the colonies grow to sufficient size to be examined. A few slides, as a makeshift, may be put in Petri dishes, the bottoms of which are covered with wet filter paper, on glass rods which keep them from getting wet. It is much more convenient, however, to have a 'moist chamber cabinet,' and when a large number of slides is used, it is quite necessary. Such a cabinet, especially designed for this work, is represented in Fig. 48*F*.

"Whatever the form of the moist chamber, it is put in an incubator and kept at the temperature best suited to the needs of the organism under study. The colonies usually attain sufficient size in the course of three or four hours to give the characteristic arrangement of the cells. If the colonies are allowed to grow much larger, the individuals become so massed that only those at the periphery are distinctly discernible, so that there is usually no advantage in large colonies.

"When the 'little plates' are removed from the incubator they are taken from the moist chamber and dried. The drying is an important step

and is apparently best done rapidly by placing the slides on a warm plate kept at a temperature just below 100° C.

Staining

"When the slides are dry, they are ready for staining. They are put first in a 10 per cent solution of acetic acid in 95 per cent alcohol for one minute or more. They are then drained and put into Loeffler's methylene blue diluted 1 : 4 with distilled water. The slides are allowed to stain from two to three minutes and are then washed free from the stain in a jar of tap water. They are dried and are then ready for microscopic examination."

CHAPTER VI

PROCEDURES FOR STUDYING THE CHARACTERISTICS OF BACTERIA

THE procedures outlined in the following pages are among the ones more commonly used. Lack of space makes it impossible to enumerate the less common ones, which may be found in other books. The "Manual of Methods for Pure Culture Study of Bacteria," prepared by the Committee on Bacteriological Technic of the Society of American Bacteriologists, should be available to students.[1] The procedures discussed below are those which may be used by the student for describing the organisms that he studies.

Invigoration of Cultures.—It would be wrong to start the study of an unknown microorganism without knowing something about its growth requirements. Some bacteria are very fastidious in their food habits. Others are unable to tolerate the presence of organic matter, and require, for cultivation in the laboratory, media prepared of pure salts. Such media are, as has been said before, termed synthetic media. Other bacteria cannot grow in the presence of oxygen. Conn proposed the following plan for invigorating cultures. The Committee of the Society of American Bacteriologists accepted it and included it in the "Manual of Methods." It is merely a logical outline for finding out some of the growth characteristics and is more significant for organisms of unknown history.

Prepare duplicate sub-cultures in standard glucose broth, and on standard agar slopes, placing cultures of each at 37° and 25° C. On the basis of the resulting growth the organism falls into one of the following series:

SERIES I: Organisms that produce good growth (surface growth, distinct turbidity or heavy precipitate) in twenty-four hours at 37° C. in glucose broth.

SERIES II: Organisms that do not produce good growth in twenty-four hours as above, but do so in forty-eight hours at 25° C. in glucose broth.

[1] Copies of this pamphlet may be purchased from Dr. H. J. Conn, Agr. Exp. Sta., Geneva, New York.

SERIES III: Organisms that do not grow well in glucose broth but do produce good growth on the surface of agar in twenty-four hours at 37° C.

SERIES IV: Organisms excluded from the above groups but which produce good growth on the surface of agar in forty-eight hours at 25° C.

It may be necessary to invigorate the culture by growing it in a specially prepared rich medium. The cultures used in an introductory course, however, may be so selected that they will not require this; only rapidly growing cultures need be used. Study of those species which grow with difficulty may be left for future courses.

Sketches.—Drawings and sketches will greatly help the student to become more observing of the growth of bacteria. They should be carefully made, since they may be used later for the identification of the same bacteria, which may be given out as unknowns. The predominating form in a stained preparation should be regarded as the typical or normal form. Attention should also be given those forms which seem to differ from the normal. Laboratory charts used in beginning courses usually provide for drawings and sketches.

STUDY OF MORPHOLOGICAL CHARACTERISTICS

Morphological characteristics are those which are related to shape and size, the presence or absence of endospores, flagella, etc. Morphological characteristics are of value, along with physiological ones, for characterizing bacteria. In a complete study of an organism both should be determined, but often only one may be of interest. Such a one may be the resistance of the spores to heat, etc.

Vegetative Cells.—The vegetative cells, in contrast with the spores, are the active, growing cells. They should, therefore, be taken from young cultures. The cells may be examined in the unstained or stained condition. With some bacteria it is difficult to secure vegetative cells without spores. When this is the case, observations may be made on the cells that contain spores.

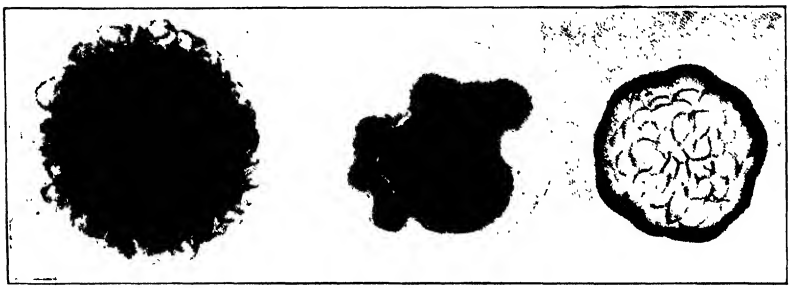
Procedure: The data required in the study of vegetative cells may be secured from stained cells or from cells in hanging drops or blocks. The cultures from which the cells are taken should be young. The following form [2] may be used for recording the

[2] Definitions of these terms will be found in Chapter VII.

data. It is taken from the Descriptive Chart of the Society of American Bacteriologists.

VEGETATIVE CELLS, Medium used.....
reaction.....temp.....age.....days.
Form, *spheres, short rods, long rods, filaments, commas, short spirals, long spirals, curved.*
Arrangement, *single, pairs, chains, fours, clusters, cubical packets.*
Limits of length.....; of diameter.....
Size of majority.....
Ends, *rounded, truncate, concave, tapering*.....
CAPSULES, present on.....
How stained.....

Where time permits, observations should be made on cells from various media as well as cells of different ages.



A *B* *C*

FIG. 50.—Forms of Bacterial Colonies.*

	A	*B*	*C*
Form	*Irregular*	*Irregular*	*Circular*
Surface	*Rough*	*Smooth*	*Rough*
Elevation	*Probably smooth*	*Raised?*	*Rough?*
Edge	*Curled*	*Lobate*	*Undulate*
Internal structure	*Curled*	*Finely granular*	*Coarsely granular*

*Attention should be called to the fact that the characterization of the colonies of bacteria shown in this and succeeding figures, is only approximate. The student may find them helpful, however, in describing the colonies of bacteria with which he works.

Abnormal Forms.—This term implies the existence of a regular or normal form. The early bacteriologists used the term " involution form " for abnormal cells. These were thought to be weak-

ened or diseased forms. Recent work on life-cycles of bacteria and other explanations of morphological changes have given these irregular forms added significance. The old "involution form" may now be interpreted in a new light and given more rational explanations.

Procedure: Study the predominating form in the microscopic field and then determine whether there are abnormal forms. The common *Azotobacter* species show many abnormal forms. Draw if desired.

A *B* *C*

FIG. 51.—Forms of Bacterial Colonies.

	A	*B*	*C*
Form	*Rhizoid*	*Conglomerate*	*Rhizoid*
Surface	*Rough*		*Rough?*
Elevation	*Effuse*	*Flat?*	*Flat?*
Edge	*Filamentous*		*Filamentous*
Internal structure	*Probably granular*		

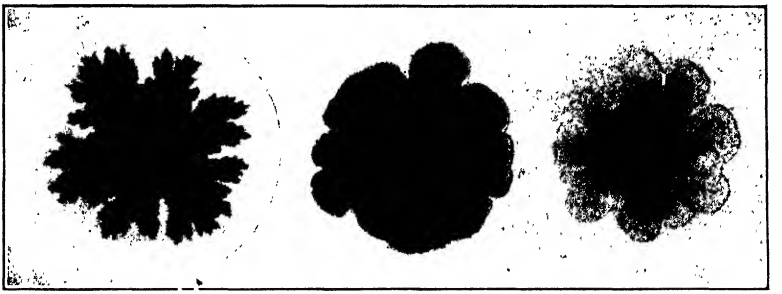
Motility.—Observations for motility should be made with hanging drops prepared with cells from young broth or agar cultures. Negative results with hanging-drop preparations are not always conclusive and several observations should always be made with cells from various media. Positive motility examinations should be followed by flagella stains.

The student must decide, oftentimes, between true motility, a distinct translocation in the field and what is called "Brownian movement." The latter is not motility of a cell but a vibratory motion due to impacts of molecules on it.

Procedure: For determining motility make a hanging-drop preparation and examine with the oil-immersion objective. If the cells appear to be non-motile or doubtfully motile, warm the slide very cautiously and examine again. If motility is still negative, remove some cells from another culture and repeat the examination. Negative observations in bacteriology always mean less than positive ones, and several observations should be made on cells of different ages and from several media before the organism is described as non-motile.

MOTILITY, In broth.....On agar.....

FLAGELLA, No.....Attachment, *polar, bipolar, peritrichiate.* How stained.....



A *B* *C*

FIG. 52.—Forms of Bacterial Colonies.

	A	*B*	*C*
Form	*Rhizoid*	*Irregular*	*Irregular*
Surface	*Rough?*	*Smooth?*	*Rough?*
Elevation	*Flat?*	*Raised?*	*Raised?*
Edge	*Erose*	*Lobate*	*Lobate*
Internal structure			

Endospores.—These are resistant stages in the life cycle of the organism. They are formed within the vegetative cells. Endospores may be demonstrated by staining reactions and by heat tests and cultures.

Procedure: First Method: The commonest method of demonstrating the presence of endospores is by the use of ordinary stained films. The spore resists the stain, just as it resists other

agents, and appears as a clearer refractile body imbedded in a more deeply stained cell. This, as far as the spore is concerned, is a sort of negative staining process. Use carbol fuchsin or methylene blue, without heating, on films prepared as described in the previous chapter.

Second Method: The other method involves the use of staining procedures by which the spore is stained. If desired, the spore may be stained one color and the vegetative protoplasm another. In accordance with our information about the resistance of the spore to unfavorable conditions, spore-staining procedures are rather rigorous. It takes considerable effort to get the dye into the cell, but once the cell is stained, it holds the dye tenaciously. Follow the procedure suggested in Chapter IV, page 93.

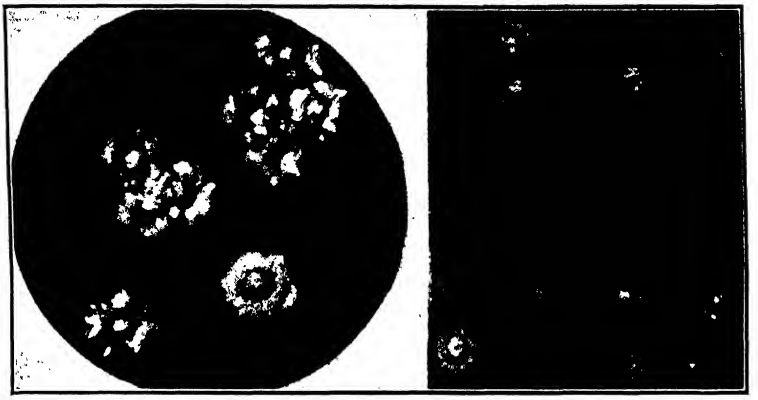
Supplementary Method: If attempts to demonstrate endospores by staining give unsatisfactory results, a heat test may be applied. For ordinary bacteria (not thermophiles) the bacteria should be suspended in sterile water and heated to 80° C. for ten to fifteen minutes; if subcultures from this tube show the presence of growth, the presence of endospores is proven because the endospores resist the heat applied above.

The Descriptive Chart of the Society of American Bacteriologists has the following convenient method for recording the data from observations on spores.

SPORANGIA, *present, absent.* Medium used.....
reaction.....temp.....age.....days.
Form, *elliptical, short rods, spindled, clavate, drumsticks.*
Limits of length.....; of diameter.....
Size of majority.....

ENDOSPORES, *present, absent.*
Method of examination, in *stained* or *unstained* preparations.
If stained, by what technic?.....
.....
Location of endospores, *central, excentric, subterminal, terminal.*
Form, *spherical, ellipsoid, cylindrical.*
Limits of size.....
Size of majority.....
Wall, *thick, thin.*
Sporangium wall, *adherent, not adherent.*

Capsules.—The presence of capsules may be indicated either by special staining procedures or by growth in the common media. The special staining procedures have been described in a former chapter. Capsulated bacteria usually grow with a sticky, slimy vegetation which, when touched with a needle, will pull out into a thread. In liquid media the sediment at the bottom of the culture tube will often show a slimy appearance. On the ordinary films stained with aqueous alcoholic solutions of the aniline dyes,



A *B*

FIG. 53.—Colonies of Bacteria on Solid Media (Agar or Gelatin).

	A	*B*
Form	*Irregular*	*Filamentous*
Surface	*Rough*	*Rough*
Elevation	*Umbilicate (umbonate)*	*Raised*
Edge	*Lobate*	*Filamentous*
Internal structure		

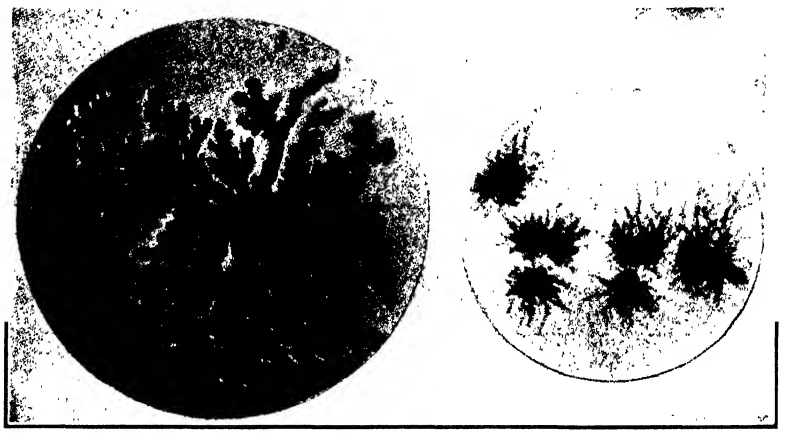
capsulated bacteria may appear with a halo about each cell. This halo represents the capsule, which seems to resist the stain. It may be made more visible by slightly moving the fine adjustment of the microscope up and down.

An organism should not be recorded as having capsules unless they have been actually stained by one of the methods of capsule staining described in Chapter IV. The Gins method, as described by Hagan, may be used if desired.

Procedure: The presence or absence of capsules should be determined by testing the growth on an agar slant for stringiness. Remove a small amount of growth from the agar slant, prepare a film and stain according to one of the procedures described in Chapter IV.

STUDY OF PHYSIOLOGICAL CHARACTERISTICS

Physiological characteristics have little to do with morphology. They are functional characteristics, depending on properties



A *B*

FIG. 54.—Forms of Bacterial Colonies.

	A	*B*
Form	*Filamentous (branching)*	*Filamentous (threadlike)*
Surface	*Rough*	*Rough*
Elevation	*Probably flat*	*Flat—spreading*
Edge	*Filamentous?*	*Filamentous?*
Internal structure		

deeply seated in the cell. These characteristics include the action of the organism on gelatin, sugars and other substances. Physiological characteristics are used together with morphological for classifying bacteria.

Cultural Characteristics.—They should be recorded from cultures which have been incubated at the optimum temperature for

the organism under study. Some bacteria, such as those pathogenic for human beings, should be incubated at 37° C., while others require 20°–25° C. For the latter, room temperature is sufficiently accurate during the cooler times of the year.

General Procedure: All inoculated cultures should be carefully labeled and placed either in the incubator or in the place designated by the instructor. Some cultures will require special incubation temperatures. These also may be announced by the instructor. The culture tubes should be examined at regular intervals and the growth recorded as scanty, moderate or abundant. (These are indefinite terms but they will help to describe the extent of growth for each individual.)

FIG. 55.—A Colony of Bacteria on Solid Media.

Form,	*Spreading.*
Surface,	*Flat.*
Elevation,	*Probably flat.*
Edge,	*Lacerate.*
Internal structure,	

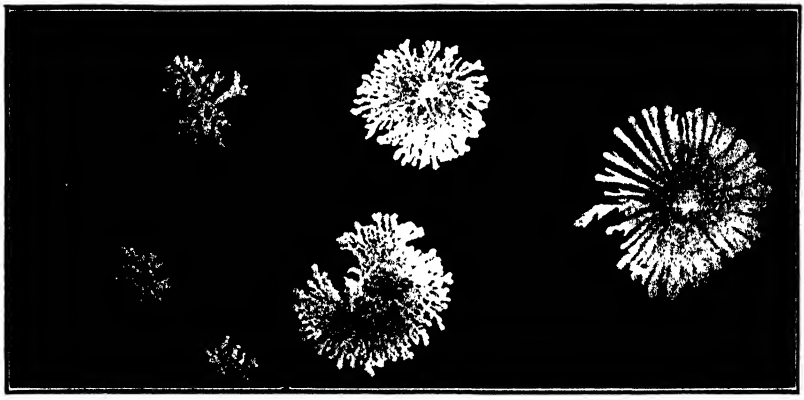
Oxygen Relations.—This determination is one of the unsatisfactory ones called for in the study of bacteria. A short, satisfactory, convenient method is difficult to find. Perhaps this is true on account of the fact that sharp lines of demarcation do not exist among groups of living organisms. It is known that even strictly anaerobic bacteria need a little oxygen, even though the amount is very small.

The methods which have been used for studying the oxygen relations fall into several groups. These include culturing the organism in evacuated containers, in containers from which the oxygen has been absorbed by aerobic bacteria, and in containers in which some of the oxygen may have been absorbed by chemicals or partially replaced by inert gases (hydrogen, nitrogen, illuminating gas, etc.). Several of these methods were described in Chapter IV.

Procedure: The Committee of the Society of American Bacteriologists proposed the following methods:

Provisional Method 1: Determine by noting the presence or

absence of growth in open and closed arm, respectively, of fermentation tubes containing glucose broth. Care must be taken to use, for inoculation, fermentation tubes from which the dissolved oxygen has been recently driven off by heating. In case of gas production, this test is of comparatively little value, because bubbles of gas may carry the sediment up with them; hence, if an organism produces gas from glucose, the test should, if pos-



A *B* *C*

FIG. 56.—Forms of Bacterial Colonies.

	A	*B*	*C*
Form	*Irregular*	*Irregular*	*Irregular*
Surface	*Rough*	*Radiately ridged*	*Radiately ridged*
Elevation	*Flat*	*Flat*	*Flat*
Edge	*Filamentous*	*Filamentous*	*Filamentous*
Internal structure			

sible, be made in the presence of some other sugar which it attacks (acidifies) without gas formation. It must be remembered, however, that even anaerobes do not grow in the absence of free oxygen except in the presence of a chemical substance (such as carbohydrate) which they are able to reduce and use as a source of oxygen.

Provisional Method 2: The agar shake culture affords a very good way of determining the oxygen requirements of an organism. A tube of about 10 c.c. of glucose agar in fluid condition (45° C.) is inoculated and agitated in such a manner that the bacteria are

evenly distributed through it. The tube should then be cooled until it has solidified and incubated. Colonies of strictly anaerobic bacteria will not grow in the upper layers of the medium where the oxygen concentration is high. On the other hand, aerobic bacteria will grow only in the upper layers.

To determine whether the organism is facultative with regard to oxygen requires both anaerobic and aerobic cultivation.

RELATION TO OXYGEN.

Method used.....

Medium.....Temperature.....°C.

Aerobic growth: *absent, present, better than anaerobic growth.*

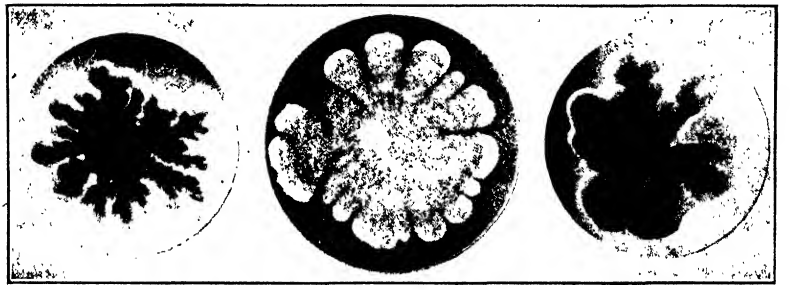
Anaerobic growth: *absent, occurs in presence of dextrose,* of *sucrose,* of *lactose,* of *nitrate; better than aerobic growth.*

Additional data.....

.....

.....

.....



A *B* *C*

FIG. 57.—Forms of Bacterial Colonies.

	A	*B*	*C*
Form	*Irregular*	*Irregular*	*Irregular*
Surface	*Rough*	*Rough*	*Rough*
Elevation	*Raised*	*Flat*	*Raised*
Edge	*Lobate*	*Lobate*	*Lobate*
Internal structure			

Staining Reactions.—The methods of staining bacteria have been given in the chapter on staining procedures. The Gram stain and one or two ordinary procedures should be applied to all

bacteria. The results may be recorded on the laboratory chart in the following form.

STAINING REACTIONS.

Gram: 1 day.....; 2 day.....; 3 day.....;
4 day.....; .....day.....Technic used.....
.....
Acid fast.....
Special stains.....
.....
.....

Nutrient Broth Cultures.—The behavior of a bacterium in nutrient broth is not only an important step in its characterization, but the use of this medium gives a convenient method for propagating it. After the nutrient-broth medium has been inoculated and incubated at the proper temperature, it should be examined carefully with as little shaking as possible. Some bacteria grow with a pellicle or ring formation which is broken and settles to the bottom when disturbed.

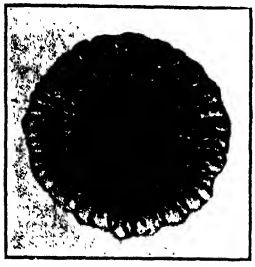


FIG. 58.—A Bacterial Colony.

Form, *Irregular.*
Surface, *Radiately ridged.*
Elevation, *Raised.*
Edge, *Entire.*
Internal structure,

Procedure: Inoculate the sterile broth tube with a loopful of the suspension or by means of the needle if a solid culture is used. Incubate and examine. Record the results by using the following form and descriptive terms, or others if it is felt that they are more suitable.

Surface growth, *ring, pellicle, flocculent, membranous, none.*
Clouding, *slight, moderate, strong, transient, persistent, none, fluid, turbid.*
Odor, *absent, decided, resembling*.....
Sediment, *compact, flocculent, granular, flaky, viscid on agitation, abundant, scant, none.*

Agar Streak.—The agar slant culture should be carefully examined to determine the characteristics of growth. This inspection should yield information on the points listed below.

Procedure: The agar slant should be inoculated with the needle. After the needle has been dipped into the culture, it should be placed at the bottom of the slanted surface and drawn up the center of the slant. Care should be exercised not to cut through the surface of the slant. It is best not to lay the agar slant flat on the desk before inoculation because the liquid that is often present at the bottom of the slant may spread over the surface. This will cause the growth to spread during incubation

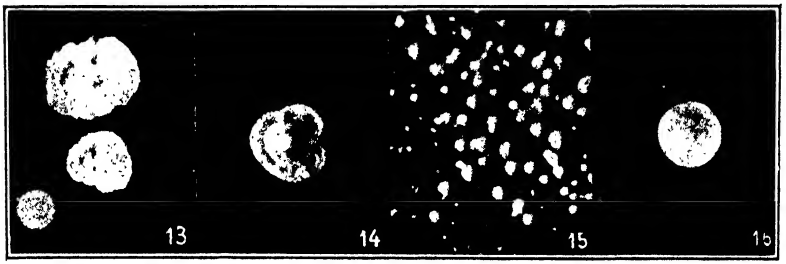


FIG. 59.—Forms of Bacteria.

	13	14	15	16
Form	*Irregular*	*Irregular*	*Punctiform*	*Punctiform*
Surface	*Rough*	*Rough*	*Smooth*	*Smooth*
Elevation	*Raised*	*Umbonate*	*Convex*	*Convex*
Edge	*Undulate*	*Undulate*	*Entire*	*Entire*
Internal structure				

and one will not secure typical growth of the organism under study. Record the results by underlining the following terms.

Growth, *scanty, moderate, abundant, none.*
Form of growth, *filiform, echinulate, beaded, spreading, arborescent, rhizoid.*
Elevation of growth, *flat, effuse, raised, convex.*
Luster, *glistening, dull.*
Topography, *smooth, contoured, rugose.*
Optical characteristics, *opaque, translucent, opalescent, iridescent.*
Chromogenesis.....photogenic, fluorescent.
Odor, *absent, decided, resembling*.....
Consistency, *butyrous, viscid, membranous, brittle.*
Medium, *grayed, browned, reddened, blued, greened.*

Agar and Gelatin Colonies.—Good agar plates should contain few colonies. The colonies should also be well isolated so that each one may grow without restriction. Spreading colonies may be avoided by the use of Hill's porous covers. The following observations should be made.

1. Examine the Petri dish to determine whether the colonies are evenly distributed and whether they are alike. Colonies with widely divergent characteristics are likely to be due to mixed cultures.

2. Compare (and draw) the colonies that are growing on the surface and those that are growing down in the medium.

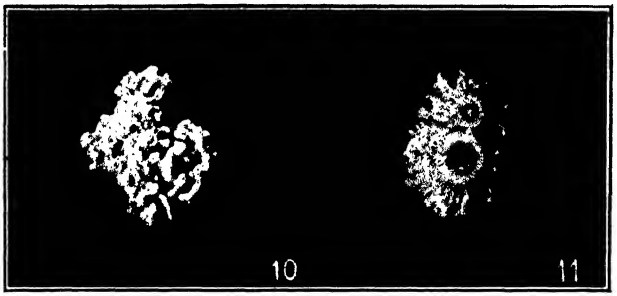


FIG. 60.—Forms of Bacteria.

	10	11
Form	*Irregular*	*Irregular*
Surface	*Rugose*	*Radiately ridged*
Elevation	*Raised*	*Crateriform*
Edge	*Irregular*	*Irregular*
Internal structure		

3. A typical surface colony and a typical deep colony should be marked for detailed study. This may be done according to the following outline.

4. Turn the Petri dish upside down and place on the stage of the microscope.[3] By means of the low-power dry objective, focus on to the edge of the colony for securing information concerning this part of the colony. If there is no further need for the Petri dish, remove the cover and place the bottom on the stage. Focus the objective down close to the colony.

[3] Low-power microscopes for just such observations are now made by several manufacturers. These magnify from 20 to 40 times and are especially useful for studying colonies. They are called stereomicroscopes by one manufacturer.

Procedure: Agar colonies for the study of characteristics should be made according to procedures outlined in the former chapter. The colonies should not be crowded. Record the results according to the following outline.[4]

Growth, *slow, rapid.*

Form, *punctiform, circular, irregular, mycelioid, filamentous, rhizoid.*

Surface, *smooth, rough, concentrically ringed, radiate.*

Elevation, *flat, effuse, raised, convex, pulvinate, umbonate.*

Edge, *entire, undulate, lobate, erose, filamentous, curled.*

Internal structure, *amorphous, finely-, coarsely-granular, filamentous, curled, concentric.*

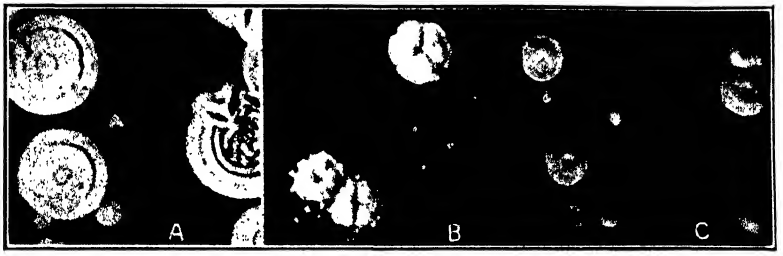


FIG. 61.—Forms of Colonies.

	A	B	C
Form	*Round*	*Ameboid*	*Round*
Surface	*Concentrically ridged*	*Rough*	*Smooth*
Elevation	*Umbonate*	*Pulvinate*	*Pulvinate*
Edge	*Entire*	*Lobate*	*Entire*
Internal structure	Amorphous	Amorphous	

Gelatin Stab.—A straight needle should be used and the tube stabbed to the bottom. It should be incubated at 20° C. and frequent observations made to determine the amount of liquefaction. The amount of liquefaction may be measured by means of a millimeter rule although the data are only approximate.

Procedure: The straight needle must be used in this preparation. Having inoculated the sterilized needle by dipping it into the culture, push it straight down into the gelatin medium and then withdraw it carefully. Incubate at 20° C., and examine

[4] The pictures of colonies shown in this chapter were taken from various papers in the Centralblatt für Bakteriologie, Part II.

regularly for liquefaction. If gelatin liquefaction is negative, record the time of incubation.

Growth, *uniform, best at top, best at bottom.*

Line of puncture, *filiform, beaded, papillate, plumose, arboresent, villous.*

Liquefaction, *none, crateriform, napiform, infundi-buliform, saccate, stratiform*; begins in.....d. complete in.....d.

Depth of liquefaction in tube of 10 mm. diameter, evenly inoculated at 20° C. for thirty days.....mm.

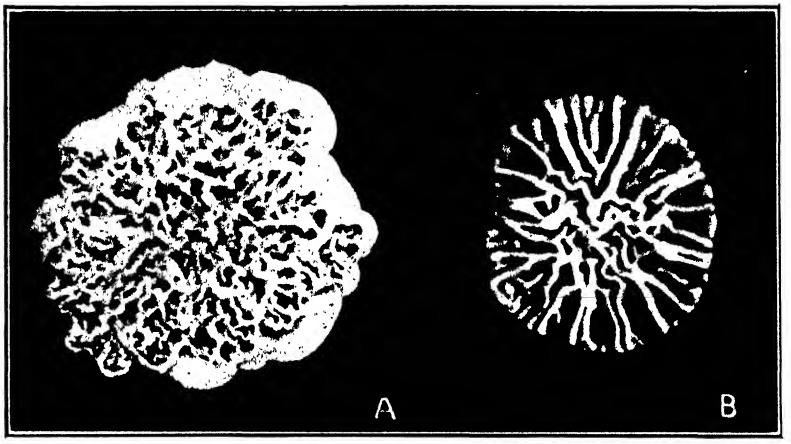


FIG. 62.—Forms of Bacterial Colonies.

	A	*B*
Form	*Irregular*	*Round*
Surface	*Rugose*	*Rugose*
Elevation	*Raised*	*Raised*
Edge	*Lobate*	*Entire*
Internal structure		

Growth on Potato and the Hydrolysis of Starch.—The inoculation of potato slants should be made in the same way as for agar slants. The potato may be tested for diastatic action according to the directions given in the paragraphs that follow. Instructions have been given before for the preparation of potato slants.

Iodine should be used to show the presence of diastatic action. Pour the contents of the culture tube into a beaker and dilute with distilling water. If dilution is sufficient and if the organism

possesses amylase, the various colors of the starch decomposition products with iodine will be obtained. Some of these are as follows:

Starch,	blue color with iodine
Soluble starch	blue color with iodine
Erythrodextrin,	red color with iodine
Achroodextrin,	no color with iodine
Maltose,	no color with iodine
Dextrose,	no color with iodine

To secure success with this method, both the iodine and starch solutions should be very dilute. The same method has been applied to potato slants. In this case, the potato slants must be thoroughly mashed and, after dilution with distilled water, treated as above.

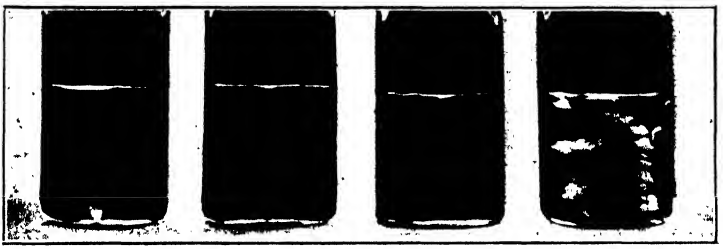


FIG. 63.—Showing Different Types of Growth in Nutrient Gelatin. (After Orla-Jensen.)

No. 35. *Filiform.* No. 5. *Filiform.*
No. 34. *Filiform.* No. 12. *Arborescent* (?)

Allen (1918) has described another convenient method which has been used by bacteriologists for some time. A 0.2 per cent thymol starch agar is melted and poured into a sterile Petri dish. After it has hardened, it is streaked with the culture under question and incubated. After incubation the plate should be flooded with dilute iodine solution. A colorless halo about the line of inoculation indicates the presence of amilase. In order to satisfy the requirements of the Descriptive Chart, Allen has stated that a clear zone of more than 2 mm. width should be regarded as *strong*, while a feeble action is denoted by a width of less than 2 mm.

Edson and Carpenter (1912), to determine the presence or absence of diastatic action, added a 2 per cent thymol starch paste

to a ten-day-old broth culture. After incubation for about eight hours, the culture was tested for reducing sugars by means of Fehling's solution.

The Committee of Standard Technique of the Society of American Bacteriologists, in one of its recent reports, made the following recommendation.

Procedure: Pour a sterile Petri dish with a tube of melted cooled[5] starch agar. This agar should contain 0.2 per cent of soluble starch. After the medium has hardened, make a streak or dot inoculation in the center of the dish with the culture under observation and incubate in the inverted position. At the end

FIG. 64.—Showing the Agar Plate with Zones of Amylolysis. (After Allen).

The clear zones along the lines of inoculation are due to the digestion of the starch by enzymes formed by the bacteria.

of the incubation period, flood the plate with a strong iodine solution. A clear zone about the growth indicates an hydrolysis of the starch.

HYDROLYSIS OF STARCH.

Breadth of clear zone on starch agar plates.

in.....days:.....

Additional data (e.g., by method of Meyer and Gottheil).....

.....

.....

[5] It is best to cool the melted agar somewhat, since this reduces the amount of moisture which condenses on the cover of the dish. If hot melted agar is poured into the sterile Petri dish a greater amount of water condenses on the cover. This may cause difficulty later by dropping on to that surface of the medium.

Temperature Relations.—The determination of this characteristic has been usually limited to 37° C. and 20° C. Other temperatures may be used as desired. The organism should be inoculated into a medium in which it will grow well. This culture should then be incubated at different temperatures. The possibility of other factors inhibiting growth should be carefully guarded against. It may be necessary to study this characteristic before starting other culture work in order to determine the optimum temperature. Negative reports on observations should always indicate the length of the incubation period.

Procedure: The temperature relations for growth may be determined in broth, upon agar slants or with other media. The culture tubes should be inoculated with the colony or suspension prepared for inoculations. The tubes should then be distributed in the various incubators or placed as the instructor advises.

TEMPERATURE RELATIONS.

Optimum temperature for growth.....°C. in.....days
Maximum temperature for growth.....°C. in.....days
Minimum temperature for growth.....°C. in.....days

Thermal Death Point.—The thermal death point of a bacterium is supposed to be that temperature above the maximum temperature at which the organism is killed under certain arbitrary conditions. Under ordinary conditions it has come to mean the time which is required to destroy the cells in a culture at a fixed temperature. Thermal death-point determinations are useful in the canning industry. The technic for careful determinations is given in the author's " Bacteriology and Mycology of Foods."

EXERCISE NO. 3: THERMAL DEATH POINT DETERMINATION

1. Prepare broth cultures of *Escherichia coli* (*Bacterium coli*) and *Bacillus subtilis*. Incubate at 37° C. over night, or until the next laboratory period.

2. Heat the culture of *Escherichia coli* in water at 55° C.; the culture of *Bacillus subtilis* should be heated at 100° C. in boiling water.

3. Prepare a Petri dish by pouring a tube of sterile melted, cooled, agar medium into the dish and allow to harden. (If the medium is cooled to about 45° C., before pouring, there will be less condensation water on the inside of the cover.) This dish may then be divided into

4 or 8 segments by marking across the bottom with a wax pencil. Each of these segments may be labeled with the time or heating period. (See Fig. 65.)

4. At intervals of three minutes, remove a loopful of the culture and inoculate a segment on the dish prepared as described in *3*. Separate agar slants may be used, but the plate method is cheaper since it requires less media and apparatus.

5. Incubate at 37° C. for twenty-four hours and record the results.

Action of Bacteria on Milk.—Plain Milk.—The proteolysis of casein may be determined by using either the tube of milk or the milk agar plate as suggested by Hastings.

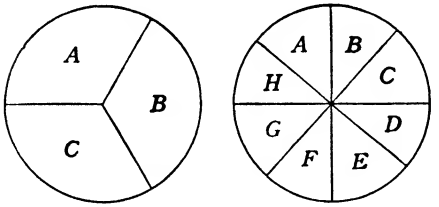


FIG. 65.—Showing the Method of Dividing a Poured Petri Dish for Experiment.

The letters should be marked on the bottom (outside) of the dish. If desired, numbers indicating the length of exposure in minutes may be used.

Procedure (Hastings' Method): Melt and cool a flask or tube of plain agar to 50° C.; add 10 per cent of sterile skimmed milk and allow to harden. After cooling, streak the pure culture under examination across the medium and incubate. Proteolytic action resulting from the pure culture will be indicated by a clear zone about the growth. To be certain that this clear zone is due to proteolysis, flood the dish with dilute acetic acid; if the clear zone remains it may be inferred that a protease was formed by the pure culture.

Frequently it is desirable to determine the reaction of the milk culture after incubation. The Committee which prepared the "Manual of Methods" suggested the use of brom-cresol purple. This may be added to the culture and compared with the color secured by adding the same quantity of indicator to sterile milk. Brom-thymol blue does not give good results in milk. Cohen, in 1923, announced another indicator, brom-chlor-phenol red (*p*H 3.2–4.8) which may be used in milk.

Four degrees of acidity that can be easily recognized in milk are listed in Table VII. They correspond closely to those listed in Table V, differing only in that brom-cresol purple is used instead of brom-thymol blue to show "neutrality," and that the curdling point (*p*H − 4.7) is used to distinguish between "moderate" and

"strong" acidity, instead of the less definite point of maximum red to methyl red. The same methods of expression used in recording acidity in clear media should be used in recording that of milk.

TABLE VII

DEGREES OF ACIDITY EASILY RECOGNIZED IN MILK

Acidity	Indicator Reactions	Approximate p^H-value
"Neutral"	Same color with brom-cresol purple * as sterile milk; i.e., blue to gray-green.	6.2–6.8
"Weak"	Color with brom-cresol purple lighter than in sterile milk; i.e., gray-green to greenish-yellow.	5.2–6.0
"Moderate"	Yellow with brom-cresol purple.† Not curdled.	4.7–5.0
"Strong"	Curdled. Blue or green to brom-phenol blue.	3.2–4.6
"Very Strong"	Yellow to brom-phenol blue.	Under 3.0

* Use a 0.04 per cent alcoholic solution.
† Or with brom-chlor-phenol red (pH, 3.2–4.8).

Litmus Milk, Purple Milk, etc.—These media differ from plain milk only in the addition of an indicator to the milk before inoculation. Until several years ago, litmus was commonly used; the preparation of better indicators for biological work has made possible the substitution of brom-cresol purple for litmus. Milk containing this indicator is often called "purple milk." The behavior of bacteria in sterile litmus milk permits grouping of bacteria in several groups depending on the type of change which they induce in the appearance of the tube. The following groups are possible.

1. Inert group.
2. Acid-forming group.
3. Acid-coagulating group.
4. Alkali-forming group.
5. Peptonizing group.

This attempt at grouping bacteria is, of course, quite arbitrary. It has, however, been useful in the study of bacteria in various dairy products.

The *inert* bacteria are those which bring about no visible change in the appearance of the litmus-milk culture tube. They apparently possess no enzymes with which to decompose the milk constituents. Before reporting an organism as inert, one should be certain that the culture tube was inoculated and every opportunity given the microorganisms for growth. Unless this is done, one might describe a bacterium which had not had a good opportunity to grow in milk as inert. In careful work this test should be applied several times with cells from different cultures.

The *acid-forming* bacteria are those which ferment the lactose in the milk to organic acids, among which lactic acid is believed to predominate. The bacteria in this group are separated from those in the acid-coagulating group, probably by the fact that they do not form enough acid to precipitate the casein. The litmus-milk tube is turned pink but still retains its liquid consistency.

The *acid-coagulating* bacteria are those which form a sufficient amount of lactic acid to precipitate the casein. The casein exists in milk as calcium caseinate. The lactic acid removes the calcium thus precipitating the casein, which is insoluble in the milk serum. If the reaction of the milk is acid and if a curd is formed, it may be regarded as an acid curd. Some acid-coagulating bacteria form gas along with the acid. This gas may appear either as bubbles throughout the curd, or it may be seen to rise in young cultures and cause a foam on the surface. Many of the anaerobic spore-forming bacteria bring about what is called a " stormy fermentation " in milk.

Peptonizing bacteria are those which possess proteolytic enzymes with which to attack the proteins in the milk. Peptonization may take place either before or after coagulation. It is indicated by a clearing and disappearance of the casein due to digestion. The terms *proteolysis* and *liquefaction* are often used synonymously with *peptonization*. When the milk is peptonized, it is changed from an opaque medium to one that is darker and clearer. This change may continue until all of the medium has become altered except a residue in the bottom of the culture tube.

Rennin curd is the curd that appears when the reaction is

neutral or slightly alkaline. In this manner, acid curd may be separated from rennin curd. When rennin acts on milk to curdle it, paracasein is formed from the casein. Bosworth stated that two molecules of paracasein were formed from one of casein. This would seem to indicate that the action of rennin was a cleavage of the casein. Bosworth believed it to be such and stated accordingly that coagulation with rennin was the first step in proteolysis.

The *alkali-forming* bacteria are those which produce an appreciable amount of alkaline substances from the milk constituents and turn the litmus in the milk to a deep blue.

Reduction, applied to litmus milk, refers to the complete loss of color of the indicator. The litmus is reduced to the colorless leuco-base. When a litmus-reducing organism is inoculated into milk the lower portion of the tube is colorless while the upper portion, down about 1 cm. or so, may be pink. The pink layer is due to absorption of the oxygen from the air. Students who are beginning their observations on cultures of bacteria in litmus milk frequently confuse reduction of the litmus with other changes.

Procedure: Inoculate a tube of sterile litmus milk or purple milk with the pure culture and incubate at the optimum temperature for the microorganism under study. In very careful work, a sterile tube of the milk should be incubated with the inoculated tube in order that minute alterations in the appearance of the medium may be better detected. This practice need not be followed in the class work unless the instructor advises. Record the results on the laboratory chart.

MILK (with or without indicator) Temperature.....°C.....

Reaction: 1 day..... 2 days.... 4 days.... 7 days.... 10 days....

Acid curd: 1 day..... 2 days.... 4 days.... 7 days .... 10 days....

Rennet curd: 1 day..... 2 days.... 4 days.... 7 days.... 10 days....

Peptonization: 1 day..... 2 days.... 4 days.... 7 days.... 10 days....

Indicator in milk (if any).....

Influence of indicator on growth.....

Reduction of litmus begins in.....days; ends in.....days; of methylene blue in.....hours.

Hydrogen Sulfide.—This compound (H_2S) is a reduced sulfur compound. It may be formed in the putrefaction of proteins or by the anaerobic reduction of sulfates.

Procedure: Suspend a strip of bibulous paper, which has been impregnated with lead acetate, in the top of the plain broth or other culture medium. The paper and lead acetate may be sterilized in the autoclav and then handled by sterile forceps. Observe whether the paper is blackened or not; if it is, hydrogen sulfide formation is indicated. The results should be recorded as follows:

PRODUCTION OF HYDROGEN SULFIDE.

Medium.....

Test used.....

H_2S *absent, present in*.....days

Another method may be employed using lead acetate agar slants. These should be inoculated in the usual manner and, after incubation, examined for blackening at the edges of growth.

Nitrate Reduction.—The utilization of nitrate, usually resulting in reduction of the nitrate, is one of the differential tests in bacteriology. This may be followed in two ways, by determining the quantity of nitrate present before and after the test, and by testing for the products of reduction, such as ammonia and nitrites. The first method requires such a long chemical analysis that it is unsuited for elementary courses. Bacteriologists, therefore, ordinarily use methods for detecting the presence of some special product of decomposition of the nitrate. Such products are nitrite and ammonia.

Procedure for Nitrites: Inoculate the pure culture into nitrate broth and on to nitrate agar slants. Incubate for forty-eight or ninety-six hours and then test for nitrite by adding 3 drops of sulfanilic acid and 3 drops of di-methyl-a-naphthylamine to each nitrate broth culture or on to each nitrate agar slant. The appearance of a deep-red color indicates the presence of nitrites. If a control tube shows no nitrite it may be assumed that the nitrite was formed from the nitrate by reduction.

Procedure for Ammonia: The use of Nessler's reagent for the detection of ammonia in bacteriological cultures was so unsatisfactory that bacteriologists have sought another method. Thomas reported a colorimetric method which may have some merit. This method was used by Ayers, Rupp and Mudge for the study of ammonia production by streptococci. The following description is taken from their report.

Reagents: A 4 per cent solution of phenol in water.
A solution of sodium hypochlorite containing 1 per cent of available chlorine.

Procedure: Two-tenths of a cubic centimeter of the medium to be tested are diluted to 8 c.c. with water, to which are added 1 c.c. of the phenol reagent and 1 c.c. of the hypochlorite reagent. These are thoroughly mixed and allowed to stand for thirty minutes. The resulting coloration (if ammonia is present) is blue in a colorless medium and green in a yellow medium.

Detection of Indol Formation.—Indol is a compound formed in the decomposition of the amino acid, tryptophan. Consequently, to secure the formation of indol, tryptophan must be present in the medium. It is probably supplied by most of the commercial peptones used in the preparation of media. It is possible, however, to have peptones that do not contain tryptophan, and thus no indol would be formed even by organisms which are known to produce it. The great number of methods for detecting indol suggest the difficulty that has been experienced. Only several of the tests which have been found to give satisfactory results will be mentioned below. The student should find out just how indol is formed from tryptophan. The chemical mechanism is given in many texts on bacteriology and biochemistry.

Procedure: Remove a portion of the broth culture of the organism, the indol-forming properties of which it is desired to determine, and proceed as directed for making the Goré test for indol.

Salkowski Test.—This should be applied as a ring test. Allow from 1 to 2 c.c. of a 25 per cent solution of sulfuric acid to run down the inside of the culture tube containing the material to be tested for indol. Then add 1 c.c. of potassium nitrite, very carefully. The formation of a pink ring where the medium comes in contact with the acid indicates the formation of indol.

Ehrlich's Test.—To 3 c.c. of culture add 1 c.c. of 2 per cent para-dimethylamidobenzaldehyde in 95 per cent alcohol, and 10 drops of conc. HCl. The proper interpretation of this test is that indol gives a *rose-red* color, soluble in chloroform, while tryptophan gives a *blue* color, insoluble in chloroform and deepening on standing for thirty hours.

Goré Modification of Ehrlich's Test.—Goré modified the Ehrlich test as follows:

1. Remove the cotton plug from the culture tube.
2. Moisten the under surface with 4–6 drops of the persulfate solution and then 4–6 drops of the para-dimethylamidobenzaldehyde solution.

3. Replace the plug and push it down to within an inch or so of the surface of the culture medium.

4. Place the tube upright in a boiling water bath and heat for fifteen minutes.

5. Remove and examine the under surface of the plug. The presence of indol is indicated by a rose color.

This test is especially suitable, for confusing color reactions are prevented. These frequently occur when the reagents are added directly to the medium.

The Oxalic Acid Test for Indol.—This test is based upon the formation of a pink color upon contact of volatile indol and dry oxalic acid crystals. Absorbent paper, such as filter paper, is dipped into an aqueous solution of oxalic acid cut into strips; one strip of such paper is then suspended from the cotton stopper in the mouth of the tube containing the culture to be tested. When indol is formed it volatilizes and turns the color of the paper pink. If no indol is formed the color remains white.

Fermentation Reactions.—The fermentation reactions usually concern the changes in carbohydrate media. The carbohydrates are added either to plain broth or to plain agar. The observations are usually made with fermentation tubes in which the broth is sterilized. These have been discussed before. Great care must be used in the sterilization of these media, since Mudge (1917) and Hasseltine (1917) have shown that poly-saccharides are hydrolyzed to mono-saccharides. The last of these investigators found that serious errors might be introduced in the study of the action of *B. proteus*, for instance, on common carbohydrates For careful work, other methods of sterilization than by heat should be used. The broth, after the carbohydrates have been added, may be sterilized by filtration, which would not introduce the error of hydrolysis of polysaccharides. This might explain why, with a few bacteria, anomalous fermentation reactions are secured.

Detection of Gas Formation.—One important product of bacterial action on carbohydrates is gas, composed usually of carbon dioxide and hydrogen. The presence of such products indicates that the bacterium is able to completely oxidize carbohydrates. Gas formation may be determined in two ways.

First Method.—This method requires the use of a fermentation tube. If gas is formed it will collect in the closed arm.

Second Method.—With care and frequent observation, gas formation may be detected with carbohydrate broths in ordinary culture tubes. The tubes are inoculated and incubated in the usual manner. If the organism is a gas former, bubbles may be seen to rise in the medium; as the fermentation progresses, a foam may appear on the surface of the medium.

Third method.—Prepare a shake culture with dextrose (etc.) agar as outlined before, and incubate. If gas is formed the culture medium will be broken up and part of it pushed up into the tube.

Fourth Method.—Inoculate the organism into dextrose (or other carbohydrate) broth. Seal the culture fluid with sterile, hot paraffin and allow to harden. If gas is formed the paraffin plug will be forced upward in the culture tube.

Acid Formation.—Some organisms are not able completely to oxidize carbohydrates but are able partially to decompose them. The main products, in such cases, are acids. Even when gas is formed, it may have to go through the acid stage.

Formerly, to determine the amount of acid formed, 5 c.c. of the culture were titrated with N/20 NaOH with phenolphthalein as the indicator. Clark and Lubs' work showed that this method was unsuitable. It is now better to determine the *p*H (hydrogen-ion concentration). The methods for this are essentially given in the chapter on media. Table V gives the indicators that may be used.

Procedure: Inoculate the several fermentation tubes with the pure culture and incubate or place where the instructor directs. At the end of the fermentation period examine for growth and the presence of gas in the closed arm. With the Smith type of fermentation, observations may also be made on the presence of growth in the closed arm this may serve to indicate whether the microorganism grows anaerobically or not. After these observations, add a few drops of brom-thymol blue to determine whether acid or alkali has developed. Or, if more accurate information is desired of the hydrogen-ion concentration, remove a portion of the fermented medium and test according to methods described before. The following form may be used for recording fermentation-tube data.

Medium containing .... and:	d-Glucose	Levulose,	d-Galactose	d-Mannose		l-Arabinose	l-Xylose	Rhamnose		Maltose	Lactose	Sucrose		Raffinose	Melezitose			Dextrin	Inulin	Glycogen
Gas																				
First appearance of acid.....																				
First appearance of alkali.....																				
Reaction after.....days																				
Reaction after.....days																				
Max. H-ion Conc.....																				

Pathogenicity to Animals.—This study is called for with certain microorganisms. It is not necessarily a part of bacteriology as covered in elementary courses. All kinds of animals may be used; the selection of an animal depends largely on the microorganism which is being studied or the material which is being examined. The more common laboratory animals are the rabbit, guinea pig, white mouse and white rat. Special work may demand the use of monkeys, goats, dogs, cats, etc. The following types of inoculations are used.

Subcutaneous Inoculation.—This is an inoculation under the skin. The skin is lifted slightly and the needle inserted.

Intraperitoneal Inoculation.—This is an inoculation into the peritoneal cavity.

Intravenous Inoculation.—The inoculation in this case is made into the veins. This is quite direct and results are secured in a shorter time.

Intramuscular Inoculation.—This inoculation is made into the muscles. In this case absorption is slow.

In Great Britain animal experimentation cannot be carried out except by license granted by the Home Secretary. In America such licenses are not required.

The Index Number.—This is a brief method for recording the salient characteristics of an organism. It is a summary of the characteristics. Before the index number should appear the genus, since members of different genera may have the same index number. It is not supposed that the index number will take the place of the name; it is useful, however, in studying a large number of strains. These may be grouped according to salient characteristics, and later names may be applied to these groups.

THE AGGLUTINATION REACTION

The agglutination reaction is used by bacteriologists for the diagnosis of disease as well as the identification of pure cultures. This reaction is more fully discussed in the author's "Bacteriology." A simplified technic for carrying out the reaction will be given here. Two procedures are available, the microscopic and the macroscopic. Two agents or factors are involved, an immune serum containing agglutinins and an antigen or suspension of bacterial cells. Ordinarily, the bacterial cells are living cells, but

dead cells may be used since the following two procedures call for suspensions of *Eberthella typhi*. These cells should be killed by heat at not over 60° C. for one hour.

The Microscopic Agglutination Reaction.—This procedure requires the use of a microscope for observing the preparations.

1. Carefully clean four concave slides or ring slides and place them on the staining board. Label them as follows:

No 1. Control (no serum).
No. 2. Normal serum.
No. 3. 1–20 Dilution.
No. 4. 1–40 Dilution.

Apply a small amount of vaseline to the rings if ring slides are used.

2. Place three clean, dry cover glasses on the staining board at the end of the slides or in forceps. If concave slides are used, apply a little vaseline to the edges of the cover glasses.

3. In the center of the cover glass for slide No. 1 place a loopful of sterile physiological salt solution and a loopful of the suspension of bacterial cells (antigen). Then quickly, but carefully, turn the cover glass over and place over the depression on the slide or ring. Press down carefully to make certain that the vaseline has thoroughly sealed the preparation. This technic is identical with the hanging-drop technic used for studying motility of bacteria.

4. Make another hanging drop by placing a drop of physiological sodium chloride solution on a slide, and add thereto by means of a sterile loop a loopful of normal serum supplied by the instructor.[6] Apply this cover glass to slide No. 2.

5. Make another hanging drop by placing one loopful of immune serum in the center of the cover glass, and add thereto 1 loopful of the bacterial suspension. Place this cover glass over the depression on slide No. 3. This gives a dilution of immune serum of 1–20 because we have diluted the 1–10 dilution with an equal amount of the bacterial suspension.

6. The last hanging drop calls for a dilution of immune serum of 1–40. How can you secure that when you have a 1–10 dilution with which to start? The fact that the antigen must be added must be kept in mind. The following procedure is suggested. Mix on another clean cover glass, with the sterilized loop, equal amounts of the 1–10 dilution of immune serum and sterile physiological sodium chloride solution. This will give a 1–20 dilution of immune serum. Now place one drop of this 1–20 dilution of immune serum in the center of the fourth cover glass and add thereto a loopful of the antigen (suspension of bacterial cells). Place the cover glass over the depression of the fourth slide and press down carefully.

7. Allow all of the slides to stand for forty-five minutes, then examine with the high-power dry lens or with the oil-immersion objective. If the latter is used, great precaution should be used to prevent pulling the cover glass from the slide as often happens if the immersion oil is thick.

[6] This serum should be diluted 1 : 10.

Discussion.—Two criteria are possible for determining agglutination with living bacteria: 1, loss of motility; and 2, clumping. When dead bacteria are used, clumping is the only criterion of agglutination.

The Macroscopic Agglutination Reaction.—The macroscopic method, unlike the microscopic, does not require the use of a microscope. This technic requires several small tubes, which should be clean and of uniform size.

1. Place seven tubes in a rack and label as follows:

No. 1. 1–20	No. 4. 1–160
No. 2. 1–40	No. 5. 1–320
No. 3. 1–80	No. 6. 1–640
	No. 7. Control (no serum)

2. Place in each of these tubes 1 c.c. of sterile physiological sodium chloride solution.

3. Add, to tube No. 1, 1 c.c. of the 1–10 dilution of immune serum. This will make 2 c.c. of 1–20 dilution.

4. Remove 1 c.c. of the mixture in tube No. 1 and add to tube No. 2 and so on. This will give dilutions from 1–20 to 1–640. Remove 1 c.c. from tube No. 6, and discard.

5. With a sterile pipette add a drop or so of the suspension of bacterial cells to each tube and incubate for six to ten hours at 37° C.

Discussion.—Positive agglutination reactions are indicated by the appearance of a precipitate at the bottom of the tubes or flakes along the side of the tubes.

Study of an Unknown Organism.—After several known organisms have been studied, unknowns may be given out, either single or mixed. Such a study will be very profitable to the student since he will have an opportunity to use the information which he has collected with known organisms for the identification of the unknown. If desired, the student may be asked to isolate organisms from various sources for identification as far as possible. In such work he should use his ingenuity and the various data which he has collected.

Suggested Procedure.—The unknown may be given as a water or physiological sodium chloride suspension; it may be given as an agar slant or suspended in milk, sterile soil, etc. In all cases, the organism should be plated out in dextrose or plain agar. A series of qualitative dilutions should be made, from which plates should be prepared. At the same time a smear should be stained

and examined for the type of organisms. This should indicate whether a pure culture has been received or whether the unknown is a mixture of two or more microorganisms. If several types seem to be present, their shape and other characteristics that may be determined from stained preparations should be recorded for reference. Then the plates which were prepared from the original sample should be incubated, and pure cultures isolated at the next laboratory hour. When these pure cultures have been secured they should be studied in the same manner that the known cultures were examined. The results should be recorded on a laboratory chart, and comparisons made with those pure cultures which have already been described in the laboratory.

If the specimen happens to be decayed fruit, sewage, water, milk, etc., more care will have to be taken to prepare the sample before plating. After plating, pure cultures must be isolated for study. Bergey's "Manual of Determinative Bacteriology" may be consulted for help in the identification of unknown bacterial cultures.

CHAPTER VII

STUDY OF YEASTS

THE methods used in the laboratory for the study of yeasts are quite similar to those used for the study of bacteria. The same apparatus may be used and, in general, the same media. The yeasts, however, grow better in a medium containing carbohydrates and a little acid. Some of these media especially suited for yeasts and molds have been described in the chapter on Media.

Since the cultural and morphological methods for the examination of bacteria have been discussed, and since these methods are applicable to the yeasts, only an outline of the observations to be made with yeasts will be given here. Special procedures for detecting ascospores in yeasts are presented here, since they differ from those procedures proving the presence of spores in bacteria.

I. Macroscopic Appearance of Growth.
   - *A.* Beer wort or dextrose broth.
   - *B.* Dextrose agar slants or wort agar slants.
   - *C.* Colonies on wort agar or gelatin.
   - *D.* Carrot slants.
   - *E.* Giant colonies on wort agar.

II. Microscopic Appearance of Cells.
   - *A.* Beer wort.
     1. Cells from young cultures.
     2. Cells from film or pellicle.
     3. Cells from sediment.
     4. Repeat observations after 30 days.
   - *B.* Wort agar or gelatin.
     1. Repeat examinations as listed for beer wort.
   - *C.* Carrot slants.
     1. Examine cells when young and also when old.

III. Examination for Sporulation and Sexuality.
   - *A.* Formation of ascospores.
   - *B.* Germination of ascospores.

IV. Temperature Relations.
  A. For budding.
  B. For sporulation.

V. Fermentation Reactions.
  A. Dextrose, levulose.

Demonstration of Ascospore Formation by Yeasts.—This is more difficult than the detection of endospores in bacteria. Several different methods have been proposed, two of which will be given below.

Hansen's Gypsum Block Method.—This is the method usually advised for demonstrating ascospores in yeast cells. However, it leaves much to be desired, since there are probably methods which yield more certain results. The plaster of Paris block may be made by pouring wet plaster of Paris into a cone of paper. After it has begun to set, the paper may be removed and the top of the cone cut off to give a flat surface on which to place the yeast cells. After the cone has been cut off, the block should be placed in a Petri dish or Esmarch dish, a little water added and the dish sterilized in the autoclav. The yeast cells should be placed on the block with a loop and allowed to remain there for thirty to forty-eight hours, when ascospores are supposed to appear in the cells if the species forms ascospores.

Maneval's Method.—Maneval noticed that cakes of compressed yeast that were kept in the ice box for six or eight days showed abundant spore formation in cells from the outer layers of the cake. The spores were easily demonstrable by mounting them in a drop of Gram's iodine solution and examining under a microscope with a magnification of 600 diameters.

Stained preparations were made as follows:

1. Spread a film in a small drop of water on a slide and dry in the air.
2. Fix by passing through the flame twelve to fifteen times.
3. Stain with hot carbol fuchsin one to three minutes.
4. Wash with water.
5. Decolorize with 5 per cent sulfuric acid for two to three seconds.
6. Wash with water.
7. Stain with methylene blue about three seconds.
8. Wash and examine.

The spores should be stained a bright red, and the vegetative protoplasm blue. Much greater abundance of spores followed the

propagation of pure cultures of yeasts on the Maneval's modification of Gorodkowa's agar. Very good spore formation took place in four to five days; in eight to twelve days 50 per cent of all cells developed spores.

Carrot slants have also been found to stimulate ascospore formation in yeast cells. The slants should be prepared in the same manner as potato slants. The slant should be inoculated with the yeast and kept at room temperature.

Staining of Yeast Nucleus.—Different methods are available for staining yeast nuclei. Several methods may be given.

1. Place the yeast cells on a glass slide and allow them to dry without heating.
2. Place the slide in a 3 per cent solution of ferric ammonium sulfate for two hours.
3. Wash in water and stain for thirty minutes in haematoxylin solution. Wash.
4. Then place the slide in the ferric ammonium sulfate for one to three minutes until good differentiation is secured.

Rayman and Kruis' Method.—The following steps should be carefully carried out.

1. Fix the yeast cells in a solution of iodine in potassium iodide and alcohol.
2. Then treat the cells with an ammoniacal iron-alum solution.
3. Stain with alizarin and decolorize with the ammoniacal iron-alum solution. The nucleus should be stained a deep red in a colorless cytoplasm.

Wager's Method.[1]

1. Transfer some of the yeast cells to Lugol's solution and allow them to remain for twenty-four hours.
2. Then wash in water, 30 per cent alcohol, 70 per cent alcohol and finally in methyl alcohol which is changed until all of the iodine has been washed out.
3. Prepare a film on a glass slide in the usual manner but do not fix with heat. A small amount of the alcohol suspension of yeast cells is placed on the slide and the alcohol allowed to evaporate; then a drop of water may be added and the yeast cells thoroughly mixed with it; the water is then allowed to dry up.
4. Place the slide in a dilute solution of Delafield's haematoxylin in water, and allow to remain for several hours.
5. Wash the cells with water and 2 per cent alum solution.
6. The nucleus should be visible as a stained body.

[1] Wager, H., The Nucleus of the Yeast Plant. Annal Bot. 12 (1898), 499–539.

Wager, H., and Peniston, A., Cytological Observations on the Yeast Plant. Ann. Bot. 12 (1898), 499; 24 (1910), 45.

Demonstration of Glycogen.—Glycogen is a reserve carbohydrate which is present in yeast cells. It may be demonstrated by adding iodine-potassium iodide solution (6 grams potassium iodide, 2 grams iodine and 120 c.c. water) to the cells. The cytoplasm of the cell will be stained brown, and the glycogen a deep red.

Demonstration of Fat in Yeasts.—Add a little of a 1 per cent solution of osmic acid to the yeast cells. The fat droplets will be stained brown.

Separation and Demonstration of Dead and Living Yeast Cells.—It is often desirable to know the relative proportion of living and dead cells in cultures of pressed yeast. The methods for determining this information are staining methods and depend on the fact that dead cells take the dye or stain while living cells do not. Several different dyes have been used. We shall use methylene blue.

1. Thoroughly clean a glass slide and a cover glass.
2. Secure an aqueous solution of methylene blue (0.1 gram in 100 c.c. of distilled water).
3. Place several loopfuls of the methylene blue on the end of the slide and place therein a little of the yeast suspension or culture.
4. Drop the clean cover glass over the preparation and allow it to stand for twenty minutes.
5. By means of the oil-immersion objective, determine the relative number of dead and living cells. Dead cells are stained blue. Living cells are unstained or nearly so.

Instead of the above method, the stain may be placed in a small test tube and the yeast suspension added. The cells are thus stained in the test tube and may then be placed on the glass slide.

EXERCISE NO. 4: STUDY OF PRESSED YEAST

1. Unwrap a cake of pressed yeast very carefully and, by means of s sterile loop or needle, remove a piece the size of a pea to a tube containing 3–5 c.c. of sterile water. Shake until an even suspension has been prepared.

2. Plate out in dextrose agar, using several dilutions. Incubate at 37° C.

3. When the plates prepared in (2) have grown (after two days at 37° C.) prepare a pure culture by transferring a colony to a sterile agar slant.

4. Examine the suspension just prepared in a hanging drop. Note the character of the material in the image.

5. Determine the cultural characteristics of this yeast by inoculation of common media.

6. Place a drop of the yeast suspension on a clean cover glass and add a drop of aqueous solution (0.5 gram in 100 c.c. of water) of methylene blue. After a few minutes, determine the number of living cells. The dead cells will take up the dye while the living cells will not.

EXERCISE NO. 5: PREPARATION OF PURE CULTURE BY LINDNER'S METHOD

1. Place a row of sterile, plugged test tubes in a test-tube rack.

2. Into each of these tubes deliver 10 c.c. of sterile water and add a small portion of pressed yeast (0.3–0.5 gram). Shake thoroughly.

3. Examine a loopful of this suspension under the microscope in a hanging-drop preparation to secure some idea of the concentration of the cells.

4. Remove a loopful from the original suspension and add it to the second tube. This should be done several times before further microscopic examinations of loopfuls are made.

5. Repeat this until microscopic examination shows not over one cell in a field. Some fields may show no yeasts.

6. Now inoculate a series of fifteen sterile tubes of beer wort each with a loopful of the diluted suspension.

7. In those that show growth the probability will be that but one cell was introduced. A pure culture has thus been secured by the single-cell method.

EXERCISE NO. 6: DEMONSTRATION OF BUDDING OF YEASTS

1. Prepare a hanging-drop preparation with a little sterile beer wort.

2. Inoculate with a small amount of yeast growth or preferably with a loopful of a yeast suspension containing but one cell.

3. Incubate and examine for budding.

EXERCISE NO. 7: TO DETERMINE THE FERMENTING POWER OF PRESSED YEAST

1. Prepare for sterilization a 100-c.c. Erlenmeyer flask with Alwood ventilation valve as shown in Fig. 67.

2. The flask should contain 40 c.c. of a 10 per cent solution of sucrose (cane sugar).

3. Sterilize the flask with sugar solution and ventilation valve in the autoclav. Immediately after removing from the autoclav, fill the ventilation valve with a sufficient concentrated sulfuric acid to seal it.

4. After cooling, inoculate with 1 gram of the compressed yeast. Replace the stopper with ventilation valve and incubate for twenty-four hours at 30° C. Weigh the flask before and after incubation.

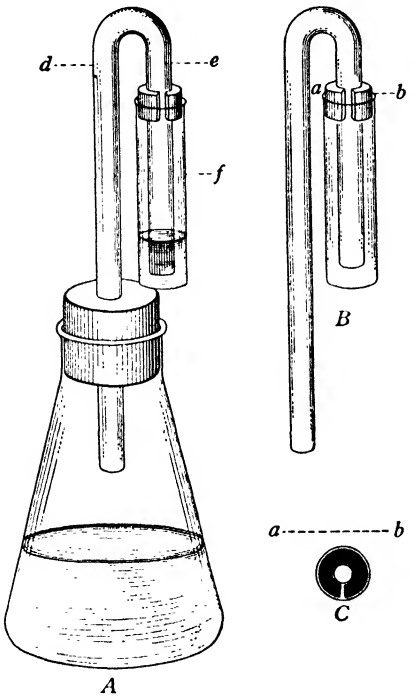


FIG. 66.—Showing a Fermentation Valve Devised by Nadson and Burgwitz.

This has the advantage over the Alwood device shown in another illustration that it is cheaper and may be made from materials which are available in even the smallest laboratories.

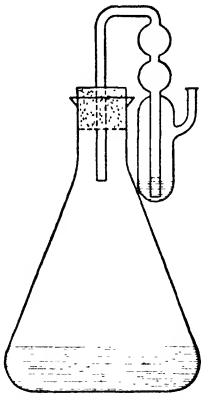


FIG. 67.—Showing an Alwood Ventilation Valve.

Sulfuric acid is placed in the valve to hold back the moisture. The carbon dioxide is allowed to escape. Loss in weight, then, is due to the escape of carbon dioxide.

EXERCISE NO. 8: TO DETERMINE THE FERMENTING ACTIVITY OF YEASTS

The work in the previous exercise may be repeated with the exception that 10 grams of yeast are used in 400 c.c. of 10 per cent sugar solution, and weighings for loss of carbon dioxide are made each half hour for five or six hours. The results may be plotted. Use the ventilation valves described in former exercises.

EXERCISE NO. 9: EFFECT OF FREEZING ON YEASTS

1. Inoculate an agar slant with a pure culture of yeast (*Saccharomyces cerevisiae*) and incubate at 30° C. over night or until the next laboratory period.

2. Add about 2–3 c.c. of sterile physiological sodium chloride to the agar slant culture and scrape off the growth by means of a sterile platinum needle.

3. Sterilize a number of 2-c.c. bacterin ampoules in the autoclav. During sterilization they may be placed upside-down in a basket in the bottom of which is a layer of cotton.

4. Fill the ampoules with about 1½ c.c. of the yeast suspension and seal immediately in the flame. A sterile syringe and needle will greatly facilitate the filling of the ampoules.

5. Determine the number of cells per cubic centimeter by diluting and plating in dextrose agar.

6. Place the ampoules in the tray of an electric refrigerator and allow to freeze.

7. At definite intervals remove an ampoule, melt at room temperature, and plate out in dextrose agar to determine the number of living cells.

8. Plot the number of living cells against the time.

REFERENCES

GUILLIERMOND, A. The Yeasts. (Trans. by F. W. Tanner.) John Wiley & Sons, Inc., New York, 1920.

CHAPTER VIII

STUDY OF MOLDS

THE type of vegetation of molds causes some slight modifications in technic; however, in most cases, the same methods may be used as for bacteria and yeasts.

The following observations may be made.

I. Macroscopic Appearance.
   A. Colonies on solid media (agar, gelatin, etc.)

II. Microscopic Appearance.
   A. Examine edges of agar colonies.
   B. Remove the cover of the dish. Examine by putting low-power dry objective into or near the growth.
   C. Remove some of the hyphae, spread in a drop of water and drop a cover glass over it. Examine.
   D. Determine how the spores are formed and the structure of the fruiting bodies.
   E. Determine whether the hyphae possess cross wall, septa, or not.

Himmel's Method for Studying Mold Mycelium.—In order to avoid the unsatisfactory method of studying mold hyphae which is now commonly used, Himmel proposed the following:[1]

Between two glass slides (5 cm. by 11½ cm.) are placed several layers of filter paper of the same size as the glass slides, the interior portions of which have been cut out so as to form a border of filter paper about 1 cm. wide. A small piece (2 or 3 cu. mm.) of the moist bread on which the culture is growing is placed between the glass slides in the center of the band of filter paper. The slides are then tied together with thread, the filter paper moistened by dipping the edges of the slides in water and the whole mount placed under a bell jar. In about two or three days the stolonifers will extend outward in various directions from the moist bread, and wherever they come in contact with the glass surface rhizoid-like hyphae and sporangiophores are produced.

[1] Himmel, W. J., The Study of Rhizopus in the General Course of Botany. Science 66 (1927), 136.

This may now be studied either with the compound microscope or with the binocular microscope.

This enables the student to trace the stolonifers with ease from their origin to their attachments to the glass and to study the sporangiophores and rhizoid-like hyphae in their natural positions without any disturbance of the hyphae or any danger of their drying during the study.

The above-described damp chamber is practically the same as that used by Dr. R. E. Jeffs in his studies of root-hair elongation and described in the *American Journal of Botany* 12: 577–606, 1925.

EXERCISE NO. 10: ISOLATION OF OIDIUM LACTIS[2]

1. Place some skimmed milk in a shallow dish and place in the incubator.

2. Allow it to sour and remain in the incubator until a velvety growth covers the surface.

3. Remove some of this growth to a little sterile salt solution. Shake and plate out in dextrose agar.

4. Isolate pure culture in the usual manner.

EXERCISE NO. 11: ISOLATION OF RHIZOPUS NIGRICANS

1. Moisten a slice of bread in a saucer and brush a little dust on to it.

2. Place the saucer and bread in a dark, humid place until the fungi have developed.

3. Prepare agar plates with some of the black or grayish mold and isolate a pure culture for further study.

EXERCISE NO. 12: OBSERVATIONS ON GERMINATION OF MOLD SPORES

1. Prepare a moist chamber with beer wort and a few mold spores. These may be secured from an old plate colony or agar slant culture.

2. Seal the moist chamber well with vaseline and hold for further observation.

3. Examine at intervals to determine type of germination.

4. Draw at regular intervals.

EXERCISE NO. 13: EFFECT OF HEAT ON MOLD SPORES

1. Prepare dextrose-broth cultures of several species of molds. Allow the cultures to grow for a week or so.

[2] Care should be used in handling cultures of molds in the laboratory. The great number of spores formed by each plant makes their dissemination easy. Old cultures and apparatus resulting from a study of molds should be sterilized in the autoclav before washing.

2. At the end of this time, shake the culture vigorously to break the spores from the fruiting bodies. Pour this suspension into a sterile plugged culture tube and hold for use. Examine in hanging drop for the presence of spores.

3. To make the test, place the culture tubes with the mold-spore suspension in a boiling water bath and, at intervals of three minutes, remove a loopful of the suspension for inoculation of a sterile dextrose agar slant. Incubate the culture tubes to determine how long the mold spores endured the heat.

EXERCISE NO. 14: THE EFFECT OF FREEZING ON MOLD SPORES

1. Inoculate several flasks of dextrose broth with pure cultures of several molds which it is desired to use in this experiment. Allow them to grow for several weeks or until a luxuriant growth has developed.

2. Shake the flask well to separate the conidia from the fruiting bodies, and strain through sterile cheesecloth to remove the large masses of mycelium.

3. Place about 1½ c.c. of the filtrate, which should contain many mold conidia, in sterile 2-c.c. ampoules and seal in the flame.

4. Place the sealed ampoules in the trays of an electric refrigerator and allow to freeze. Place an accurate thermometer in one of the trays in order to determine the exact temperature that is reached.

5. At frequent intervals remove an ampoule from each lot, allow to melt at room temperature and plate out in dextrose agar. The initial count should have been determined before the experiment was started.

6. Plot the results.

CHAPTER IX

PLAN OF WORK

LABORATORY work in bacteriology is somewhat different from that in the physical as well as some of the biological sciences. The student of bacteriology works with living organisms and consequently must make observations at regular intervals. In order to have cultures with which to work at subsequent laboratory periods, it is necessary to prepare them several days in advance. It is very difficult to record in advance just what will be done at each class period. Interruptions due to factors beyond control often arise. Some classes progress more rapidly than others. However, the author has found it not only desirable but essential for thorough work to have an outline toward which to work. This is just as beneficial for the student.

Below is given a plan of work for a class meeting three times a week on alternate days. This is perhaps the best sequence of class periods because it leaves a sufficiently long time interval for growth and multiplication of common microorganisms to take place in the cultures between class periods. The intervals are, also, not too long, and therefore do not cause growth to go too far. However, where such a sequence of class periods is impossible other programs may be arranged.[1] That presented here works well if plating is done on the same period each week, although this is not strictly necessary. Where this practice is followed, however, in large laboratory classes, it makes possible the regular preparation of supplies and materials and thus helps to standardize the work in the preparation room.

The outline presented in the following pages is based upon a study of a number of pure cultures of bacteria and special exer-

[1] The author would be glad to assist any who might desire it, in the preparation of an outline of work for courses meeting at other intervals and lengths of time than those considered in this book.

cises or experiments. The study of the pure cultures involves both morphological and physiological observations. Such work is necessary for giving experience in the methods for determining the various characteristics. Good technic in the bacteriological laboratory is acquired only by practice. The special exercises are included to illustrate some of the basic characteristics of micro-organisms as well as to break up the monotony of cultural and morphological studies.

A. First Day

1. Inoculate the following culture media from a representative plate colony.[2]

a. Nitrate broth, page 144.
b. Nutrient broth, page 132.
    1. Hydrogen sulfide test, page 144.
c. Gelatin stab, page 135.
d. Agar slant, page 133.
e. Dextrose fermentation tube, page 147.
f. Lactose fermentation tube, page 147.
g. Sucrose fermentation tube, page 147.
h. Glycerol fermentation tube, page 147.
i. ..... fermentation tube.
j. Litmus milk.
k. Starch agar plate, page 138.
l. Potato slant, page 136.

2. Observe and draw macroscopic and microscopic appearance of agar plate colony prepared at the previous laboratory period.

[2] If the plate colonies are small, a suspension may be prepared by removing a colony from the plate to a small amount of sterile water in a culture tube. This should be thoroughly broken up. Then a loopful of this suspension may be used for inoculation of the culture media. In this way a great number of inoculations may be made from one colony and much time saved. Otherwise one would have to wait for a broth or agar culture to develop.

Note: On page 167 is given a list of experiments and exercises. The dates in the first column indicate when the experiments are started and the dates in the second column when the reports are due. Such a list is posted on the bulletin board in the author's laboratory. One is prepared at the beginning of each semester. This one is included here as a sample.

B. Second Day

1. Make observations and drawings on the following cultures:
   Nutrient broth culture, page 132.
   Gelatin stab culture, page 135.
   Agar slant culture, page 133.
   Potato slant culture, page 136.
2. Make the required observations on the litmus milk culture.
3. Make required observations and drawings on the following:
   Agar plate colonies, page 135.
   Gelatin plate colonies, page 135.
4. Make required observations on fermentation tubes.

4a. Make hanging drop preparation.

5. Make stains as follows:
   Gram stain, page 89.
   Aqueous-alcoholic stain, page 86.
   Special stains when necessary.
6. Examine cells for endospores, capsules shape, etc.
7. Start work with special exercise.
8. Make observations on special exercises started at the last period.

C. Third Day

1. Observe and draw the following:
   Nutrient broth culture, page 132.
   Gelatin stab culture, page 135.
   Agar slant, page 133.
2. Make required observations on litmus milk and record, page 141.

2a. Make required observations on fermentation tubes.

3. Make and record the final observations on the following:
   Agar plate colonies.
   Gelatin plate colonies.
4. Divide nutrient broth culture into three equal portions. Dilute with ammonia-free water and test these separate portions for ammonia, nitrite, and indol formation, page 145.
5. Divide nitrate broth culture into two parts and test for ammonia and nitrites, page 144.
6. Complete any work that has been left from previous periods.
7. Determine diastatic action on starch agar plate, page 138.
8. Prepare agar plates with cultures for next week's laboratory work.
9. Continue or complete work on Special Experiment.

Name	Course
Date	Lab. Desk No.

Experiment No.

Object:

Apparatus:

Procedure:

Results:

Conclusions:

References:

BACTERIOLOGY LABORATORY UNIVERSITY OF ILLINOIS URBANA, ILLINOIS	Report Submitted

DEPARTMENT OF BACTERIOLOGY

Bacteriology 5b — 1*st Semester*, 1927–28

1. Use of Microscope	Sept. 23	Sept. 23
2. Preparation of beef-extract broth	Sept. 26	
3. Preparation of beef-extract agar	Sept. 28	
4. Microscopic examination of bacteria and staining	Sept. 30	Oct. 5
5. Spore stain	Oct. 3	Oct. 5
6. Negative stain	Oct. 5	Oct. 7
7. Capsule stain	Oct. 5	Oct. 7
8. Flagella stain	Oct. 7	Oct. 7
9. Types of bacteria in air, dust, etc	Oct. 7	Oct. 12

Study of Individual Types of Bacteria and Special Experiments

10. *Bacillus Ramosus*	Oct. 12	Oct. 21
11. Spore-former from dust or air	Oct. 19	Oct. 28
12. *Bacillus megatherium*	Oct. 19	Oct. 28
13. *Sarcina lutea*	Oct. 26	Nov. 4
14. *Micrococcus candidus*	Oct. 26	Nov. 4
15. Effect of high temperatures on bacteria	Oct. 28	Nov. 4
16. Effect of drying on bacteria	Oct. 31	Nov. 9
17. *Bacterium coli*	Nov. 2	Nov. 11
18. *Bacterium capsulatum*	Nov. 2	Nov. 11
19. Action of disinfectants	Nov. 4	Nov. 9
20. Effect of sunlight on bacteria	Nov. 7	Nov. 9
21. Unknown for morphology	Nov. 9	Nov. 14
22. *Proteus vulgaris*	Nov. 9	Nov. 18
23. *Bacterium alcaligenes*	Nov. 9	Nov. 18
24. Effect of dyes on bacteria	Nov. 14	Nov. 18
25. *Serratia marcescens*	Nov. 16	Nov. 23
26. *Corynebacterium pseudodiphtheriticum*	Nov. 16	Nov. 23
27. Effect of metals on bacteria	Nov. 18	Nov. 23
28. Unknown for morphology	Nov. 21	Nov. 23
29. Examination of milk	Nov. 30	Dec. 5
30. Unknown for identification (1)	Nov. 30	Dec. 9
31. Acid-fast stain	Dec. 2	Dec. 5
32. Examination of water	Dec. 7	Dec. 16
33. Unknown for identification (2)	Dec. 9	Dec. 21
34. Anaerobic bacteria	Dec. 16	Dec. 21
35. Granule stain	Dec. 19	Dec. 21
36. Study of yeasts	Jan. 6	Jan. 20
37. Study of molds	Jan. 16	Jan. 20

FIRST WEEK

First Meeting: Registration.

. . . . . .

. . . . . .

. . . . . .

. . . . . .

. . . . . .

. . . . . .

. . . . . .

Second Meeting: Check apparatus in lockers.

General discussion of apparatus with demonstrations by the instructor.

Discussion of conduct in the laboratory and hygiene of the laboratory.

. . . . . .

. . . . . .

. . . . . .

. . . . . .

. . . . . .

Third Meeting: Study of the microscope. Exercise No. 1, Page 45.

. . . . . .

. . . . . .

. . . . . .

. . . . . .

. . . . . .

. . . . . .

. . . . . .

SECOND WEEK

First Meeting: Finish the exercise on the microscope.

Complete drawings made in exercise on the microscope.

Preparation of beef-extract broth. Page 57.

. . . . . .

. . . . . .

. . . . . .

. . . . . .

Second Meeting: Preparation of beef-extract agar. Page 60.

. . . . . .

. . . . . .

. . . . . .

. . . . . .

. . . . . .

. . . . . .

Third Meeting: Finish all back work.

Microscopic examination of bacteria.[3]

Exercise 15, page 196.

a. Preparation of film for staining. Page 85.

b. Simple stain. Page 86.

c. Gram stain. Page 89.

d. Examination of stained preparations for spores, shape of the cells, etc.

e. Hanging drop preparation for motility. . . . . .

. . . . . .

[3] In this work the student will become familiar with some of the general procedures which will be used later on pure cultures and unknowns. Three or four organisms are used in order to include the various characteristics with which a student should become familiar.

THIRD WEEK

First Meeting: Finish back work on microscopic examination of bacteria. Spore stain.

.....

.....

.....

.....

.....

Second Meeting: Negative or relief staining with *Spirillum* and an Actinomycete.

(Burri's India ink preparation.)

Make a capsule stain.

.....

.....

.....

.....

.....

Third Meeting: Flagella staining. Use method on page.....

Determination of types of bacteria in air, dust, etc. Exercise No. 33, page 204.

.....

.....

.....

.....

.....

.....

FOURTH WEEK

First Meeting: **Finish back work.**

Study the plates made in special experiment at the previous meeting.

.....

.....

.....

.....

.....

.....

Second Meeting: .....

.....

.....

.....

.....

.....

.....

Third Meeting: Study *Staphylococcus albus* according to Outline A. (Instructor will discuss the observations and the recording of data on charts.)

.....

.....

.....

.....

.....

.....

FIFTH WEEK

First Meeting: Study *Staphylococcus albus* according to Part B on page 164.

.....

.....

.....

.....

.....

.....

Second Meeting: Finish observations on *Staphylococcus albus* according to Outline C on page 164.

Plate out the coccus isolated from air, dust, etc., and *Sarcina lutea.*

.....

.....

.....

.....

.....

Third Meeting: Study organisms plated at the last period according to Outline A on page 164.

.....

.....

.....

.....

.....

.....

.....

SIXTH WEEK

First Meeting: Study organisms plated at the last period according to Outline B on page 164.

.....

.....

.....

.....

.....

.....

Second Meeting: Complete study of the two bacteria studied at the last hour according to Outline C on page 164.

Plate out *Bacillus subtilis* and the spore-forming rod isolated from air.

.....

.....

.....

.....

.....

Third Meeting: Special Exercise No. 3, page 139. Effect of High Temperatures on Bacteria (Thermal death points).

Continue study of two organisms plated at last hour according to Outline A on page 163.

.....

.....

.....

.....

.....

SEVENTH WEEK

First Meeting: Continue the study of *Bacillus subtilis* and spore-former from air according to Outline B on page 164.

Record observations on cultures from the special experiment of the last laboratory period.

Special Experiment No. 17. Effect of Drying on Bacteria.

Second Meeting: Continue the study of *B. subtilis* and spore-former from air according to Outline C on page 164.

Plate out *Escherichia coli* and *Proteus vulgaris.*

.....

.....

.....

.....

.....

.....

Third Meeting: Start study of *Proteus vulgaris* and *Escherichia coli* according to Outline A on page 164.

Special Exercise No. 19. Effect of Disinfectants on Bacteria.

.....

.....

.....

.....

EIGHTH WEEK

First Meeting: Continue the study of *Proteus vulgaris* and *Escherichia coli* according to Outline B on page 164.

Finish all back work.

Make observations on Special Exercise started at last hour.

Special Exercise No. 18. Effect of Sunlight on Bacteria.

Continue Experiment on Effect of Drying on Bacteria.

. . . . . .

. . . . . .

. . . . . .

Second Meeting: Complete the study of *Proteus vulgaris* and *Escherichia coli* according to Outline C on page 164.

Make observations on Special Exercise No. 17. Effect of Sunlight on Bacteria.

Complete Special Exercise No. 197. Effect of Drying on Bacteria.

Plate out *Serratia marcescens* and *Klebsiella capsulatus* on plain agar.

Plate out unknown culture for morphological study.

Third Meeting: Start the study of *Serratia marcescens* and *Klebsiella capsulatus* according to Outline A on page 164.

Study unknown for morphology.

. . . . . .

. . . . . .

. . . . . .

. . . . . .

NINTH WEEK

First Meeting: Continue the study of *Serratia marcescens* and *Klebsiella capsulatus* according to Outline B on page 164.

Special Exercise No. 32. Effect of Dyes on Bacteria.

.....

.....

.....

.....

Second Meeting: Complete the study of *Serratia marcescens* and *Klebsiella capsulatus* and the unknown, following Outline C on page 164.

Make observations on Special Exercise No. 32 started at last hour.

Plate out *Alcaligines fecalis* and *Pseudomonas fluorescens*.

.....

.....

.....

Third Meeting: Start study of *Alcaligines fecalis* and *Pseudomonas fluorescens*, according to Outline A on page 163.

.....

.....

.....

Written Examination.

.....

.....

.....

TENTH WEEK

First Meeting: Continue study of *Alcaligines fecalis* and *Pseudomonas fluorescens* according to Part B of outline on page 164.

Special experiment announced by instructor.

.....

.....

.....

.....

.....

Second Meeting: Finish study of *Alcaligines fecalis* and *Pseudomonas fluorescens*, according to Part C of outline on page 164.

.....

.....

.....

.....

.....

.....

Third Meeting: Vacation.

.....

.....

.....

.....

.....

ELEVENTH WEEK

First Meeting: Exercise No. 16, page 196. Bacteriological Examination of Milk.

Start study of the unknown organism.

.....

.....

.....

.....

.....

Second Meeting: Special Exercise No. 36, page 205. Bacteria on Coins and the Oligodynamic Action of Metals.

Finish observations on Exercise 16.

Exercise 20, page 199, Bacteriological Examination of Water.

Continue work on unknown organism.

.....

.....

.....

Third Meeting: Examine cultures made at the last laboratory period with Special Exercise.

Continue observations on Exercise 20.

Continue work on unknown organism.

.....

.....

.....

TWELFTH WEEK

First Meeting: Anaerobic method and study of *Clostridium sporogenes.* Make anaerobic plates and agar shake culture; dextrose broth and litmus milk sealed with paraffin. Make a dextrose-agar slant for aerobic incubation.

Continue observations on Exercise 20.

Finish, if possible, unknown organism.

.....

.....

.....

Second Meeting: Make observations on cultures prepared with *Clostridium sporogenes.*

Finish observations on Exercise 20.

Last date for report of unknown organism.

.....

.....

.....

Third Meeting: Plate out in dextrose agar, *Saccharomyces cerevisiae, ellipsoideus, Willia belgica* and *Torula lactis.*

Make dextrose agar slants of these yeasts and store in locker for future observations for ascospores.

.....

.....

.....

.....

.....

THIRTEENTH WEEK

First Meeting: Make inoculations and observations on cultures prepared with the four yeasts plated at the last hour. Follow Outline A on page 163.

Start Special Exercise No. 4, page 155. Study of Compressed Yeast.

Prepare plates from yeast cake as directed.

Stain the nucleus in yeast cells.

.....

.....

.....

Second Meeting: Continue observations according to Outline B on page 164, with the three yeast cultures.

Make necessary observations on special experiment started at the last hour.

Make inoculations according to Outline A on page 163 with pure culture from pressed yeast.

.....

.....

.....

Third Meeting: Complete the observations on the four pure culture yeasts.

Prepare agar slant cultures of the four or five mold cultures.

Continue observations on pure culture isolated from pressed yeast.

Plate out sour milk mold. Special Exercise No. 10, page 106. Isolation of *Oidium lactis*.

FOURTEENTH WEEK

First Meeting: Isolate a culture of *Oidium lactis* from plates prepared at the last period.

Examine the pure cultures of mold secured at the last hour. Determine the genus of each.

Special Exercise No. 11, page 160. Isolation of *Rhizopus nigricans.*

.....

.....

.....

Second Meeting: Continue observations on mold cultures.

Special Exercise No. 13, page 160. Effect of Heat on Mold Spores.

.....

.....

.....

.....

.....

Third Meeting: Make observations on special experiment started at the last laboratory period.

Complete the identification of the mold cultures.

.....

.....

.....

.....

.....

.....

FIFTEENTH WEEK

First Meeting: Make examination for ascospores in the three pure culture yeasts. Use the agar slants prepared before. Follow Maneval's Method as outlined on page 153.

.....

.....

.....

.....

Second Meeting: Finish all back work.

Write up all reports.

Assemble all reports, notes, etc., and bind for submitting to the instructor.

.....

.....

.....

.....

Third Meeting: Practical examination.

1. Morphological examination of unknown culture.
   - *a.* Gram stain.
   - *b.* Presence of spores.
   - *c.* Shape of the cells.
   - *d.*

Clean out locker and return apparatus.

Invoice locker and check apparatus.

.....

.....

.....

.....

Name of organism.....Source.....Studied by.....Culture No.....

Date of isolation.....Habitat.....Optimum conditions: Media.....Temp.....°C.

Is phase variation observed?.....Phase on this Chart: S, R, M, G (smooth, rough, mucoid, gonidial).....Phases recorded on other charts:.....

Underscore required terms.

VEGETATIVE CELLS: Medium used.....
Reaction (pH).....Temp.....Age.....d
Size of Majority.....
Ends, *rounded, truncate, concave, tapering*
MOTILITY: In broth..... On agar.....
SPORANGIA and ENDOSPORES: *present, absent.*
Medium used.....pH.....Temp.....Age.....d
Endospore Form: *spherical, ellipsoid, cylindrical*
IRREGULAR FORMS:
Present on.....in.....days at.....°C.

AGAR COLONIES: Temperature.....°C. Age.....d.
Form, *punctiform (i. e. under 1 m.m. diam.), circular (i. e. over 1 m.m. diam.), filamentous, irregular, rhizoid.*
Surface, *smooth, rough, concentrically ringed, radiately ridged.*
Edge, *entire, undulate, lobate, erose, filamentous, curled.*
Elevation of growth, *effuse, flat, raised, convex.*
Optical Characters, *opaque, translucent, opalescent, iridescent.*

GELATIN COLONIES: Temperature.....°C. Age.....d.
Form, *punctiform, circular, irregular, filamentous.*
Elevation, *flat, raised, convex, pulvinate, crateriform (liquefying).*
Edge, *entire, undulate, lobate, erose, filamentous, curled.*
Liquefaction, *cup, saucer, spreading.*
Surface, *smooth, contoured, rugose.*
Optical Characters, *opaque, translucent, opalescent, iridescent.*

AGAR STROKE: Temperature.....°C. Age.....d.
Growth, *scanty, moderate, abundant, none.*
Form of growth, *filiform, echinulate, beaded, spreading, arborescent, rhizoid.*
Lustre, *glistening, dull.*
Chromogenesis.....*photogenic, fluorescent.*
Odor, *absent, decided, resembling.....*
Consistency, *butyrous, viscid, membranous, brittle.*
Medium, *grayed, browned, reddened, blued, greened, unchanged.*

NUTRIENT BROTH: Temperature.....°C. Age.....d.
Surface growth, *ring, pellicle, flocculent, membranous, none.*
Clouding, *slight, moderate, strong, transient, persistent, none, fluid turbid, granular growth.*
Odor, *absent, decided, resembling.....*
Sediment, *compact, flocculent, granular, flaky, viscid.*
Amount of sediment, *abundant, scanty, none.*

GELATIN STAB: Temperature.....°C. Age.....d.
Growth, *uniform, best at top, best at bottom.*
Line of puncture, *filiform, beaded, papillate, villous, arborescent.*
Liquefaction, *none, crateriform, infundibuliform, napiform, saccate, stratiform:* begins in.....d. complete in.....d.
Degree of liquefaction in.....days.
Method used.....
Medium, *fluorescent, browned, unchanged.*

SKETCHES

Surface Colonies | Deep Colonies

Surface Colonies | Deep Colonies

Medium: Temperature .....°C. Age .....d.

Medium: Temperature .....°C. Age .....d.

Medium: Temperature .....°C. Age .....d.

FERMENTATION Temperature.....°C.

Medium..... containing..... .....and:	Monosaccharides: Arabinose, Rhamnose, Xylose, Glucose, Fructose, Galactose, Mannose	Disaccharides: Lactose, Sucrose, Maltose, Trehalose, Melibiose, Cellobiose, *	Polysaccharides: Raffinose, Melezitose, Starch, Inulin, Dextrin, Glycogen	Alcohols: Glycerol, Erythritol, Arabitol, Adonitol, Mannitol, Sorbitol, Dulcitol	Glucosides: Salicin, Aesculin, Coniferin, 2-Methyl Gluc.
Gas in fermentation tube					
Amt. CO_2 in Eldredge tube					
Reaction (PH) after.....d.					
Titrable acidity in ml. of N/.....NaOH					

BRIEF CHARACTERIZATION

As each of the following characteristics is determined, indicate in proper marginal square by means of figure, as designated below. In case any of these characteristics are doubtful or have not been determined, indicate with the letters U, V, and X according to the following code:

U, undetermined; V, variable; X, doubtful.

MORPHOLOGICAL

Vegetative Cells

Form & arrangement: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinæ; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous

Diameter: 1, under 0.5μ; 2, between 0.5μ and 1μ; 3, over 1μ

Gram stain: 0, negative; 1, positive

Flagella: 0, absent; 1, peritrichic; 2, polar; 3, present but undetermined

Capsules: 0, absent; 1, present

Chains (4 or more cells): 0, absent; 1, present

Sporangia: 0, absent; 1, elliptical; 2, short rods; 3, spindled; 4, clavate; 5, drumsticks

Endospores: 0, absent; 1, central to excentric; 2, subterminal; 3, terminal

CULTURAL

Agar Stroke

Growth: 0, absent; 1, abundant; 2, moderate; 3, scanty

Lustre: 1, glistening; 2, dull

Agar Colonies

Form: 1, punctiform; 2, circular (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled; 6, irregular

Surface: 1, smooth; 2, contoured; 3, rugose

Gelatin Colonies

Form: 1, punctiform; 2, circular (over 1 mm.); 3, irregular; 4, filamentous

Surface: 1, smooth; 2, contoured; 3, rugose

PHYSIOLOGICAL

Biologic relationships: 1, pathogenic for man; 2, for animals but not for man; 3, for plants; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic

Relation to free oxygen: 1, strict aerobe; 2, facultative anaerobe; 3, strict anaerobe; 4, microaerophile

In nitrate media: 0, neither nitrite nor gas; 1, both nitrite and gas; 2, nitrite but no gas; 3, gas but no nitrite

Chromogenesis: 0, none; 1, pink; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, black

Other photic characters: 0, none; 1, photogenic; 2, fluorescent; 3, iridescent

Indole: 0, negative; 1, positive

Hydrogen sulfide: 0, negative; 1, positive

Hemolysis: 0, negative; 1, positive

Methemoglobin: 0, negative; 1, positive

Protein Liquefaction or Digestion

Gelatin: 0, negative; 1, positive

Casein: 0, negative; 1, positive

Egg albumin: 0, negative; 1, positive

Blood serum: 0, negative; 1, positive

Indicator Reduction

Litmus: 0, negative; 1, positive

Methylene blue: 0, negative; 1, positive

Janus green: 0, negative; 1, positive

Reaget production: 0, negative; 1, positive

SUPPLEMENTARY DATA

TEMPERATURE RELATIONS

Medium ..... pH .....
Optimum temperature for growth ..... °C.
Maximum temperature for growth ..... °C.
Minimum temperature for growth ..... °C.
THERMAL DEATH POINT: Time 10 minutes: ..... °C.
Medium ..... pH .....

THERMAL DEATH TIME:
Medium ..... pH .....

Temp.	Time	Temp.	Time
.....°C.	.....min.	.....°C.	.....min.
.....°C.	.....min.	.....°C.	.....min.
.....°C.	.....min.	.....°C.	.....min.
.....°C.	.....min.	.....°C.	.....min.
.....°C.	.....min.	.....°C.	.....min.

CHROMOGENESIS

Gelatin .....
Agar .....
Potato .....
.....

OTHER PHOTIC CHARACTERS

Photogenesis on .....
Iridescence on .....
Fluorescence in .....
.....

RELATION TO REACTION (pH) OF MEDIUM

Medium .....
Optimum for growth: *about pH* .....
Limits for growth: *from pH* ..... *to* .....

RELATION TO FREE OXYGEN

Method .....
Medium ..... Temp. .....°C.
Aerobic growth: *absent, present, better than anaerobic growth, micro-aerophilic*
Anaerobic growth: *absent, occurs in presence of glucose, of sucrose, of lactose, of nitrate; better than aerobic growth*
Additional data: .....
.....
.....

MILK

Temperature .....°C.

Reaction:	.....d. ..... ;	.....d. ..... ;	.....d. .....
Acid curd:	.....d. ..... ;	.....d. ..... ;	.....d. .....
Rennet curd:	.....d. ..... ;	.....d. ..... ;	.....d. .....
Peptonization:	.....d. ..... ;	.....d. ..... ;	.....d. .....

LITMUS MILK

Temperature .....°C.

Reaction:	.....d. ..... ;	.....d. ..... ;	.....d. .....
Acid curd:	.....d. ..... ;	.....d. ..... ;	.....d. .....
Rennet curd:	.....d. ..... ;	.....d. ..... ;	.....d. .....
Peptonization:	.....d. ..... ;	.....d. ..... ;	.....d. .....

Reduction of litmus *begins in* .....*days, ends in* ..... *days*

ACTION ON ERYTHROCYTES

Cells: .....
Method: *plate, broth, filtrate*
Hemolysis: *negative, positive*
Methemoglobin: *negative, positive*

PRODUCTION OF INDOLE

Medium .....
Test used .....
Indole *absent, present in* .....*days*

PRODUCTION OF HYDROGEN SULFIDE

Medium .....
Test used .....
H_2S *absent, present in* .....*days*

ACTION ON NITRATES

Medium ..... Temp. .....°C.

Nitrite:	.....d. ..... ;	.....d. ..... ;	.....d. .....
Gas (N_2):	.....d. ..... ;	.....d. ..... ;	.....d. .....

Medium ..... Temp. .....°C.

Nitrite:	.....d. ..... ;	.....d. ..... ;	.....d. .....
Gas (N_2):	.....d. ..... ;	.....d. ..... ;	.....d. .....

Ammonia production (in amino-N-free nitrate medium): *negative, positive*
Complete disappearance of nitrate in .....medium: *negative, positive*
Disappearance of 2 p.p.m. nitrite in .....medium: *negative, positive*

REDUCTION OF INDICATORS

Medium ..... pH ..... Temp. .....°C.

Indicator	Conc.	Reduction:	
.....	.....%	.....hr. ..... ;	.....hr. .....
.....	.....%	.....hr. ..... ;	.....hr. .....
.....	.....%	.....hr. ..... ;	.....hr. .....
.....	.....%	.....hr. ..... ;	.....hr. .....

STAINING REACTIONS

Gram: .....d. ..... ; .....d. ..... ; .....d. ..... ; .....d. .....
Method .....
Spores: Method .....
Capsules: Method .....
Medium .....
Flagella: Method .....
Special Stains: .....
.....
.....

ADDITIONAL TESTS

Methyl red: *negative, positive*
Voges-Proskauer: *negative, positive*
Growth in sodium citrate: *absent, present*
Growth in uric acid: *absent, present*
Hydrolysis of starch: *complete (iodine colorless); partial (iodine reddish-brown); none (iodine blue)*
Nitrogen obtained from the following compounds: .....
.....
.....
.....

PATHOLOGY

ANIMAL INOCULATION

Medium used ..... Age of culture ..... Amount ..... Incubation period .....

		Whole culture			Cells			Filtrate		
Animal										
Type of Injection	Subcutaneous	*								
	Intraperitoneal									
	Intravenous									
	Per os									

*In each instance where pathogenicity is observed, indicate location of lesion, and type, e. g. edema, histolysis, gas, hemorrhage, ulcer, diphtheritic, etc.

ANTIGENIC ACTION

Animal ..... Medium used ..... Age of culture .....
Type injection ..... Number of injections .....
Culture causes production of *cytolysins, agglutinins, precipitins, antibodies.*
Specificity: Antibodies produced effective against other antigens as follows .....
.....
Immune sera from .....
.....
..... effective against this organism as antigen

SPECIAL TESTS

This DESCRIPTIVE CHART presented at the annual meeting of the SOCIETY OF AMERICAN BACTERIOLOGISTS, Dec. 28, 1934, by the Committee on Bacteriological Technic.
Prepared by a sub-committee consisting of M. W. Jennison and H. J. Conn.

The outline which has just been presented is not intended to be final, but to suggest how work in an elementary course in bacteriology can be coördinated. No plan can be followed exactly, for various things arise to cause variations in plans. An outline may be posted in the laboratory showing when experiments will be started and when reports are due. This will help to keep the work moving more evenly according to schedule.

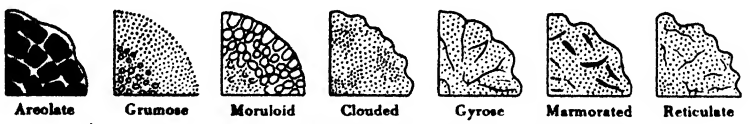
Laboratory Charts.—These are desirable on account of the fact that considerable time is saved the student and the instructor when he inspects the reports. Many different laboratory charts have been prepared during the past few years. The latest development of an official chart is the Descriptive Chart of the Society of American Bacteriologists,[4] a copy of which is inserted in this chapter.

The attempt to record characteristics of bacteria in a concise manner has resulted in the development of a series of terms which are usually quoted in books on bacteriological technic. Some of these are difficult to define and just as difficult to interpret later. For lack of a better system, those that are used on the Descriptive Chart of the Society of American Bacteriologists are given below. These terms are not the only ones that may be used. Others may be introduced, if desired.

[4] Copies of this chart may be purchased from Dr. H. J. Conn, Agricultural Experiment Station, Geneva, N. Y.

TYPES OF SURFACE ELEVATION OF COLONIES

TYPES OF MARGIN OF COLONIES



TYPES OF INTERNAL STRUCTURE OF COLONIES

FIG. X.

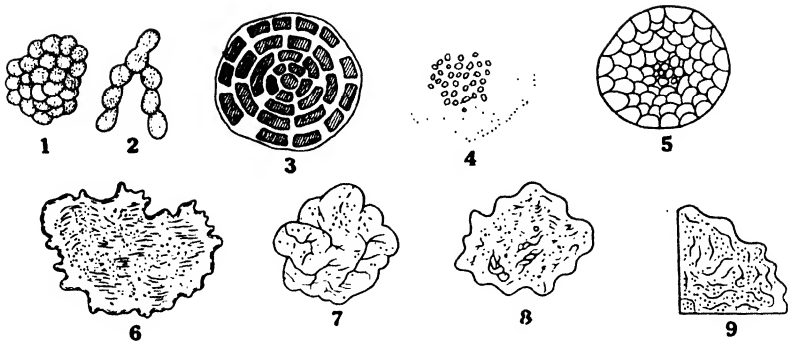


FIG. X.—Structure of Colonies.

1, Conglomerate colony; 2, toruloid colony; 3, alveolate structure; 4, grumose in center; 5, mormoid; 6, clouded; 7, reticulate; 8, marmorated; 9, gyrose.

(From Moore's Laboratory Directions for Beginners in Bacteriology, Ginn & Co. 1905.)

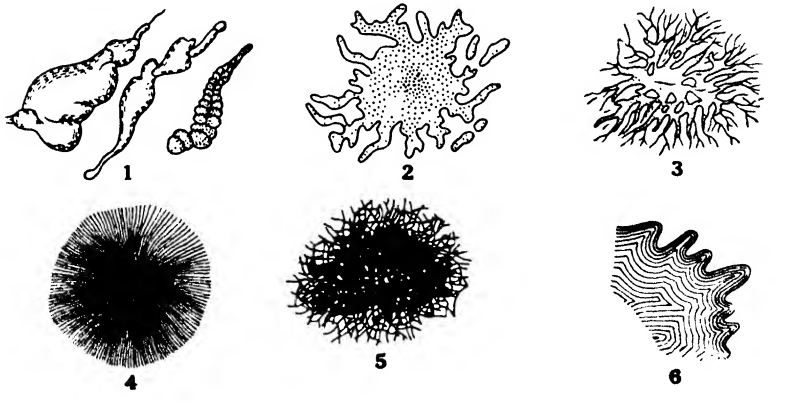


FIG. Y.—Types of Colonies.

1, Cochleate; 2, amœboid; 3, rhizoid; 4, mycelioid; 5, filamentous; 6, curled structure.

(From Moore's Laboratory Directions for Beginners in Bacteriology, Ginn & Co., 1905.)

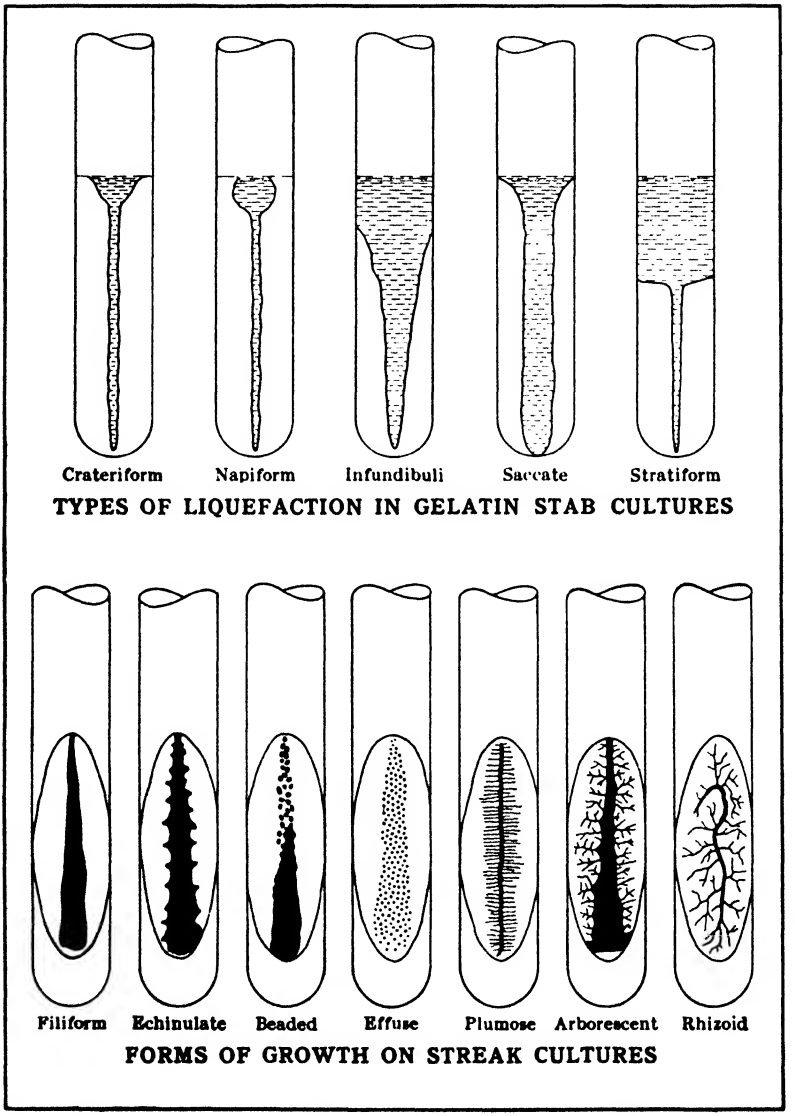

Crateriform
Napiform
Infundibuli
Saccate
Stratiform
TYPES OF LIQUEFACTION IN GELATIN STAB CULTURES
Filiform
Echinulate
Beaded
Effuse
Plumose
Arborescent
Rhizoid
FORMS OF GROWTH ON STREAK CULTURES

FIG. Z.

GLOSSARY OF TERMS USED FOR RECORDING CHARACTERISTICS OF BACTERIA

The following terms may be helpful for recording the characteristics of bacteria. If they are not, the student may use terms of his own. The character of bacterial growth is such that no set of terms exactly fits all cases.

Abundant—must be thought of in connection with the terms **scant** and **moderate.**

Adherent—applied to sporangium wall, indicates that remnants of sporangium remain attached to endospore for some time.

Aerobic—growing in the presence of free oxygen; **strictly aerobic,** growing *only* in the presence of free oxygen.

Amorphous—without visible differentiation in structure.

Anaerobic—growing in the absence of free oxygen; **strictly anaerobic,** growing *only* in the absence of free oxygen; **facultative anaerobic,** growing both in presence and absence of free oxygen.

Arborescent—branched, tree-like growth.

Beaded—(in stab or stroke culture) disjointed or semi-confluent colonies along the line of inoculation.

Bipolar—at both poles or ends of the bacterial cell.

Brittle—growth dry, friable under the platinum needle.

Bullate—growth rising in convex prominences like a blistered surface.

Butyrous—growth of butter-like consistency.

Chains—four or more bacterial cells attached end to end.

Chromogenesis—the production of color.

Ciliate—having fine, hair-like extensions, resembling cilia, **sometimes not** visible to the naked eye.

Clavate—club-shaped.

Clostridium—a spindle-shaped organism.

Cloudy—said of fluid cultures which do not contain flocculent masses.

Coagulation—the separation of casein from whey in milk.

Contoured—having an irregular, smoothly undulating surface, like that of a relief map.

Convex—surface the segment of a sphere.

Coriaceous—growth tough and leathery, not yielding to the platinum loop or needle.

Crateriform—a saucer-shaped liquefaction of the medium.

Cretaceous—growth opaque and white, chalky.

Cuneate—wedge-shaped.

Curled—composed of parallel chains in wavy strands, as in anthrax colonies.

Diastatic action—conversion of starch into simpler carbohydrates, such as dextrins or sugars, by means of diastase.

Dull—not glossy or glistening.

Echinulate—a growth along line of inoculation with toothed or pointed margins.

Effuse—growth thin, veily, unusually spreading.

Endospores—thick-walled spores formed within the bacterial cell, i.e., typical bacterial spores like those of *B. anthracis* or *B. subtilis*.

Entire—with an even margin.

Erose—border irregularly toothed.

Filaments—applied to morphology of bacteria, refers to threadlike forms, generally unsegmented; if segmented, to be distinguished from chains (q.v.) by the absence of constrictions between the segments.

Filamentous—growth composed of long, irregularly placed or interwoven threads.

Filiform—in stroke or stab cultures, a uniform growth along line of inoculation.

Film—floating islands or growth of bacteria on surfaces of liquid media. See **pellicle** and **scum.**

Fimbriate—border fringed with slender processes larger than filaments.

Flaky—similar to flocculent.

Floccose—growth composed of short, curved chains variously oriented.

Flocculent—containing adherent masses of bacteria of various shapes floating in the culture fluid.

Fluorescent—having one color by transmitted light and another by reflected light.

Granular—composed of small granules.

Glistening—glossy, not dull.

Grumose—clotted.

Infundibuliform—having the form of a funnel or inverted cone.

Iridescent—exhibiting changing rainbow colors in reflected light.

Lacerate—having a margin which looks as if it had been torn.

Lobate—having a margin deeply undulate, producing lobes. See **undulate.**

Luminous—glowing in the dark, phosphorescent.

Maximum temperature—temperature above which growth does not take place.

Membranous—growth thin, coherent, like a membrane.

Minimum temperature—temperature below which growth does not take place.

Moderate—must be thought of in connection with the terms **scant** and **abundant.**

Mycelioid—colonies having the radiately filamentous appearance of mold colonies.

Napiform—liquefaction in form of a turnip.

Opalescent—resembling the color of an opal.

Opaque—impervious to light rays.

Optimum temperature—temperature at which growth is most rapid.

Papillate—growth beset with small nipple-like processes.

Pellicle—bacterial growth forming either a continuous or an interrupted sheet over the culture fluid.

Peptonization—rendering curdled milk soluble by the action of peptonizing enzymes.

Peritrichiate—covered with flagella over the entire surface.

Persistent—lasting many weeks or months.
Plumose—a fleecy or feathery growth.
Polar—at the end or pole of the bacterial cell.
Pulvinate—decidedly convex, in the form of a cushion.
Punctiform—very small, but visible to naked eye; under 1 mm. in diameter.
Radiate—showing ray structure.
Raised—growth thick, with abrupt or terraced edges.
Reduction—removing oxygen from a chemical compound. Refers to the conversion of nitrate to nitrite, ammonia, or free nitrogen, and to the decolorization of litmus.
Rhizoid—growth of an irregular branched or root-like character, as in *B. mycoides*.
Ring—growth at the upper margin of a liquid culture, adhering to the glass.
Rapid—developing in twenty-four to forty-eight hours.
Rugose—wrinkled.
Saccate—liquefaction in form of an elongated sac, tubular, cylindrical.
Scant—must be thought of in connection with the terms **moderate** and **abundant.**
Scum—see **film.**
Slow—requiring five or six days for development.
Spindled—larger at the middle than at the ends. Applied to sporangia, refers to the forms frequently called **clostridia.**
Sporangia—cells containing endospores.
Spreading—growth extending much beyond the line of inoculation, i.e., several millimeters or more.
Stratiform—liquefying to the walls of the tube at the top and then proceeding downward horizontally.
Striate—striped.
Transient—lasting a few days.
Translucent—somewhat transparent.
Truncate—ends abrupt, square.
Turbid—cloudy with flocculent particles; i.e., cloudy plus flocculence.
Unbonate—having a button-like raised center.
Undulate—border wavy, with shallow sinuses.
Verrucose—growth wart-like with wart-like prominences.
Villous—growth beset with hair-like extensions.
Viscid—growth follows the needle when touched and withdrawn; sediment on shaking rises as a coherent swirl.
Wrinkled—small ridges formed.

BACTERIA

Name of student — Desk No.

Name of organism — Isolated from

Method of Isolation

Occurrence

Importance

Arrangement, *single, pairs, chains, fours, cubical packets.* — Size

Involution Forms

Motility — Flagella No. .....Attachment, *polar, bipolar, lophotrichiate, peritrichiate.* How Stained.....

Vegetative Cells,
Medium used.....
temp.....
age.....days.
Form, *spheres, short rods, long rods, filaments, commas, short spirals, long spirals, spindled, cuneate, clavate, curved.*

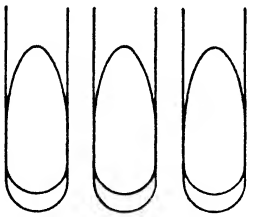
Endospores, *present, absent.*
Location of Endospores, *central, polar.*
Form, *spherical, elliptical, elongated.*
Limits of Size.....
Size of Majority.....
Wall, *thick, thin.*
Sporangium wall, *adherent, not adherent.*
Germination, *equatoria, oblique, polar, bipolar, by stretching, by absorption of spore wall.*

Capsule

Staining Reactions.
1:10 watery fuchsin, gentian violet, carbol fuchsin, Loeffler's alkaline methylene blue.
Special Stains
Gram.....
Glycogen.....
Fat.....
Acid fast.....
Neisser.....
Metachromatic granules, sporogenous granules

PLAIN AGAR STREAK

.....days .....days .....days

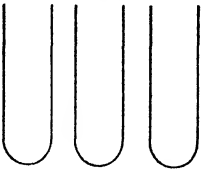


Reaction....
Incubated at
.....°C.

Growth, invisible, scanty, moderate, abundant.
Form of Growth, filiform, echinulate, beaded, spreading, plumose, arborescent, rhizoid.
Elevation of Growth, flat, effuse, raised, convex.
Lustre, glistening, dull, cretaceous.
Topography, smooth, contoured, rugose, verrucose.
Optical Characters, opaque, translucent, opalescent, iridescent.
Chromogenesis.....
Odor, absent, decided, resembling .....
Consistency, slimy, butyrous, viscid, membranous, coriaceous, brittle.
Medium grayed, browned, reddened, blued, greened.
Note.—Underline terms applying to description of cultures.

PLAIN GELATIN STAB

.....days .....days .....days



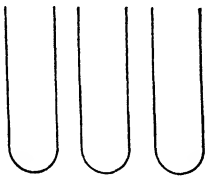
Reaction....
Incubated at
.....°C.

Growth uniform, best at top, best at bottom.
Line of Puncture, filiform, beaded, papillate, villous, plumose, arborescent.
Liquefaction, crateriform, napiform, infundibuliform, saccate, stratiform; *begins in*.....d, *complete in*.....d.
Medium fluorescent, browned.....

PLAIN BROTH CULTURE

Reaction....

Incubated at .....°C.



Surface growth, ring, pellicle, flocculent, membranous, none.

Clouding, slight, moderate, strong; transient, persistent; none; fluid, turbid.

Odor, absent, decided, resembling .....

Sediment, compact, flocculent, granular, flaky, viscid on agitation, abundant, scant.

Age of colony	.... .days	.... .days	.....days
Size of colony (Millimeters)			
Surface elevation			

AGAR COLONY

Reaction....

.....°C.

	Macroscopic	Microscopic*	Macroscopic	Microscopic	Macroscopic	Microscopic
Deep colony						
Surface colony						

* Use low power objective.

Growth, slow, rapid, temperature .....

Form, punctiform, round, irregular, ameboid, mycelioid, filamentous, rhizoid.

Surface, smooth, rough, concentrically ringed, radiate, striate.

Elevation, flat, effuse, raised, convex, pulvinate, umbonate.

Edge, entire, undulate, lobate, erose, lacerate.

Internal structure, amorphous, finely, coarsely-granular, grumose, filamentous, floccose, curled.

Age of colony	... days	.. days	.....days
Size of colony (Millimeters)			
Surface elevation			

GELATIN COLONY

Reaction....

Incubated at .....°C.

	Macroscopic	Microscopic*	Macroscopic	Microscopic	Macroscopic	Microscopic
Deep colony						
Surface colony						

* Use low power objective.

Growth, slow, rapid.

Form, punctiform, round, irregular, ameboid, mycelioid, filamentous, rhizoid.

Elevation, flat, effuse, raised, convex, pulvinate, crateriform (liquefying).

Edge, entire, undulate, lobate, erose, lacerate, fimbriate, filamentous, floccose, curled.

Liquefaction, cup, saucer, spreading.

		.....days	.....days	.....days
Litmus milk	No change			
	Acid			
	Gas			
	Acid curd			
	Rennin curd			
	Reduction			
	Alkali			
	Peptonization			

FERMENTATION REACTIONS

Per Cent of Gas In	Control	Dextrose	Lactose	Sucrose	Glycerol			
24 hours								
48 hours								
4 days								
7 days								
Total gas production								
Odor								
Acid								
Growth in closed arm								

Production of	NH_3 from peptone	
	H_2S from peptone	
	Nitrites from peptone	
	Indol from peptone	
Reduction of nitrate to	NH_3	
	Nitrites	
Diastasic action on starch	Present	
	Absent	
Chromogenesis on	Nutrient broth	
	Nutrient gelatin	
	Nutrient agar	

Index No.* . . . . . .

BRIEF CHARACTERIZATION

As each of the following characteristics is determined, indicate in proper marginal square by means of figure, as designated below. In case any of these characteristics are doubtful or have not been determined, indicate with the letters U, V, and X according to the following code:

U, undetermined V, variable X, doubtful

REMARKS:

PRIMARY CHARACTERISTICS	Microscopic Features		Form: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinæ; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous	
			Endospores: 0, absent; 1, central; 2, excentric to terminal	
			Flagella: 0, absent; 1, peritrichic; 2, polar	
			Gram stain: 0, negative; 1, positive	
	Miscellaneous Biochemical Reactions		Biologic relationships: 1, pathogenic for man; 2, for animals but not for man; 3, for plants; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic	
			Relation to oxygen: 1, strict aerobe; 2, facultative anaerobe; 3, strict anaerobe	
			Gelatin liquefaction; 0, negative; 1, positive	
			In nitrate media: 0, neither nitrite nor gas; 1, both nitrite and gas; 2, nitrite but no gas	
			Chromogenesis: 1, fluorescent; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, pink; 0, none	
	Carbohydrate Reactions		Diastatic action; 0, negative; 1, positive	
			From *d*-glucose: 0, no acid; 1, acid and gas; 2, acid without gas	
			From lactose: 0, no acid; 1, acid and gas; 2, acid without gas	
			From sucrose: 0, no acid; 1, acid and gas; 2, acid without gas	
SECONDARY CHARACTERISTICS	Vegetative Cells		Diameter: 1, under 0.5μ; 2, between 0.5μ and 1μ; 3, over 1μ	
			Length: 1, less than 2 diameters; 2, more than 2 diameters	
			Chains (4 or more cells): 0, absent; 1, present	
			Capsules: 0, absent; 1, present	
	Spores		Shape: 1, spherical; 2, ellipsoid to cylindrical	
			Diameter: 1, less than diameter of rod; 2, greater than diameter of rod	
	Cultural Features	Agar Stroke	Growth: 0, absent; 1, abundant; 2, moderate; 3, scanty	
			Lustre: 1, glistening; 2, dull	
			Surface: 1, smooth; 2, contoured; 3, rugose	
			Agar colonies: 1, punctiform; 2, circular (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled; 6, irregular	
			Gelatin colonies: 1, punctiform; 2, circular (over 1 mm.); 3, irregular; 4, filamentous	
		Milk	Acid: 0, no acid; 1, sufficient for curdling; 2, insufficient for curdling	
			Rennet curd: 0, absent; 1, present	
			Peptonization: 0, absent; 1, present	
			Indole production: 0, negative; 1, positive	
(Optional spaces)				

CHAPTER X

SPECIAL EXERCISES

THE following special exercises may be used for supplementing the routine study of microorganisms and for attaining perfection in technic. Each exercise will bring out a new technic or some special information. Some of the exercises are outlined in sufficient detail; others will have to be supplemented by directions given by the instructor. It is impossible to outline exercises in such a manner that they will be suitable without change for all laboratories. The experiments may be written up on any type of report sheet. That shown on page 165 is offered as a suggestion.

EXERCISE NO. 15: CHARACTERISTICS OF A KNOWN ORGANISM.

This experiment may be utilized during the early part of the course to demonstrate the proficiency of the student in the determination of some of the common morphological characteristics of a pure culture. The pure culture may be distributed either on an agar slant or in plain broth. Determine the following characteristics:

1. Shape of the cell.
2. Motility.
3. Presence or absence of spores.
4. Reaction to Gram stain.

EXERCISE NO. 16: BACTERIOLOGICAL EXAMINATION OF MILK

1. The sample of milk to be examined should first be thoroughly mixed.

2. Label three sterile Petri dishes as follows: (1) 1 : 100; (2) 1 : 1000; and (3) 1 : 10,000.

3. With a sterile pipette transfer 1 c.c. of milk to a bottle containing 99 c.c. of sterile water. Shake thoroughly. This will give a dilution of 1 to 100, and each cubic centimeter of the dilution will contain 0.01 c.c. of the original milk.

4. After thoroughly mixing, transfer 1 c.c. of this dilution to the Petri dish labeled 1 : 100. Then transfer in a similar way 1 c.c. to a tube of 9 c.c. sterile water. This will give a dilution of 1 : 1000.

5. Mix and transfer 1 c.c. of the 1 : 1000 dilution to the Petri dish with this label and 1 c.c. to another tube with 9 c.c. sterile water for a dilution of 1 : 10,000.

6. Repeat the above procedure to give a Petri dish with the last dilution. Into the three Petri dishes pour agar, rotate gently to mix, and incubate at 37° C. until the next period.

7. After incubation an estimate of the number of bacteria per cubic centimeter of milk is made by counting the colonies on a plate and multiplying this figure by the dilution. For accurate results, a plate should contain between 50 and 250 colonies.

Several forms of apparatus for counting plates will be demonstrated.

EXERCISE NO. 17: EFFECT OF DRYING ON BACTERIA

1. Prepare a thin suspension in sterile water of the organism to be tested. (*Serratia marcescens*).

2. Place a loopful of the suspension in the bottom of each of two sterile Petri dishes. Mark the spot on the *under* side of the plate with a wax pencil.

3. Into one of the plates pour a tube of agar (cooled to 45° C.). This plate should then be kept in the desk for a later comparison with the other.

4. The other plate, containing only the film of bacteria, should be put in the 37° incubator where it will be allowed to dry for one week.

5. After one week this will be tested to determine effect of drying. Place a loopful of sterile water on the dried film, rub up in the water and then pour a tube of melted agar into the dish. Incubate until the next period and compare with the plate that was not subjected to drying. Have all the bacteria been killed by drying? If not, what is the comparative number of colonies on the two plates?

EXERCISE NO. 18: EFFECT OF SUNLIGHT ON BACTERIA

1. Melt a tube of nutrient agar, cool to about 45° or 50° C. and inoculate from a broth culture of *Bacterium coli* or other non-spore-forming organism.

NOTE: In making this inoculation care must be taken not to get the agar so heavily seeded that distinct colonies will not be easily seen after incubation. By using the straight inoculating wire, instead of the loop, enough material should be carried over from the broth culture into the melted-agar tube to give the desired number of colonies.

2. Pour into a sterile Petri dish, rotate gently to mix and allow the agar to harden. Invert the dish and paste on the bottom a strip of dark, heavy paper. This will protect part of the plate while the rest will be exposed.

3. Expose the plate, bottom side up, to direct sunlight for two hours. Then incubate until the following period.

5. Make a record of results, either by description or drawing.

EXERCISE NO. 19: ACTION OF DISINFECTANTS ON BACTERIA

1. Use broth cultures of a spore-forming and a non-spore-forming organism which have been prepared at a former exercise.

2. The following disinfectants will be used:

Mercuric chloride:

0.1% (1 : 1000)
0.02% (1 : 5000)
0.01% (1 : 10,000)

Phenol:

5.0% (1 : 20)
1.0% (1 : 100)
0.2% (1 : 500)

Hydrogen peroxide—undiluted:

10.0% (1 : 10)
2.0% (1 : 50)

3. Into sterile test tubes plugged with cotton, measure 5 c.c. of disinfectant solution, using a separate tube for each dilution of disinfectant.

4. To each tube of disinfectant add, by means of a sterile pipette, 0.2 c.c. of broth culture of the organism to be tested. Immerse in a water bath at 20° C. After 5, 15, and 30 minutes from the time of inoculation, transfer a loop of the disinfectant culture mixture to tubes of sterile broth. These tubes should be properly labeled with the time, disinfectant used and organism tested. They are then incubated until the next period.

5. At the following period examine the broth tubes for evidence of growth. The results secured with various dilutions of each disinfectant and for each organism will be tabulated and discussed. (Note the cup plate method for determining disinfecting value described on page 206.)

EXERCISE NO. 20: BACTERIOLOGICAL EXAMINATION OF WATER

Different samples of water may be examined by members of the class. Results will be tabulated and discussed by the instructor.

Collection of Samples.—Use sterilized bottles which will be supplied and collect the sample as soon as possible before analysis. If analysis will be delayed, keep the sample in the refrigerator. Great care should be used in collecting the sample to prevent contamination.

PROCEDURE

First Period.

1. Carefully remove the stopper of the sample bottle and flame the mouth before removing the sample.

2. *Standard Agar Plate Count, Bacteria per Cubic Centimeter.*—By means of a sterile 1 c.c. pipette, place 1 c.c. of the original sample and dilutions 1–10 and 1–100 in a series of three sterile Petri dishes. Pour a tube of melted cooled agar medium into each dish and after thoroughly mixing water and medium, allow to harden. Incubate these plates in the inverted position at 37° C., until the next laboratory period.

3. *Presumptive Test. Detection of the Presence of Escherichia coli or Aerobacter aerogenes.* Into each of three tubes of ordinary lactose broth place 1 c.c. of the original sample and of the 1–10, and 1–100 dilutions prepared above. (The instructor may advise other dilutions depending on the quality of water being examined.) Label all tubes and incubate at 37° C., until the next laboratory period.

Second Period.

1. Count the colonies on the plates prepared at the last laboratory period.

2. Examine the lactose broth tubes prepared at the last exercise and record the presence of gas formation. Presence of gas formation in any of the tubes is a *positive presumptive test* for *Escherichia coli*. Absence of gas is a *negative presumptive test;* no further work is then necessary. (*See page* 297, *Tanner's Bacteriology.*)

3. Presence of gas formation in any of the fermentation tubes requires confirmation to determine whether gas formation is really due *to Escherichia coli*. (Why?)

Partially Confirmed Test.

Select the fermentation tube showing gas formation with the smallest amount of water which was used. From this, according to instructions given on page 113, streak either a plate of solidified Endo's or eosin-methylene-blue agar. Incubate in the inverted position until the next period. These media were devised to show the presence of *Escherichia coli* among other bacteria which have developed in the fermentation tube. This organism forms deep red colonies, frequently with a metallic sheen,

on Endo's agar; on eosin-methylene-blue agar, the colonies are dark with a black center. (This will be amplified by the instructor.) The presence of such colonies on these media constitutes what is called the *partially confirmed test.*

Third Period.

Confirmed Test.

1. Examine the special plates made at the last exercise for the presence of colonies which may be *Escherichia coli.* If such are found circle them on the bottom of the dish with a wax pencil. From a well isolated colony inoculate an agar slant and a lactose broth tube. Incubate at 37° C. until the next laboratory period. Also inoculate a tube of methyl-red (M.R.-V.P.) medium and a tube of citrate medium.

Fourth Period.

1. Examine the lactose broth tubes made at the last period and record presence or absence of gas formation.

2. Make a Gram stain of the growth on the agar slants and examine carefully for spores; also record whether the cells are Gram positive or negative.

3. Examine the citrate medium tubes for presence of growth.

4. Separate the methyl-red medium tubes into two parts.

a. To one part add three drops of methyl-red indicator solution. (A red color indicates *Escherichia coli*; a yellow color *Aerobacter aerogenes.*)

b. To the other part add 5 cc. of a 10% solution of potassium hydroxide. (This is the Voges-Proskauer test.) A positive test is indicated by an eosin pink color. *Escherichia coli* gives a negative test.

Note: Escherichia coli is a non-sporulating, Gram negative, lactose-fermenting rod.

EXERCISE NO. 21: TO DETERMINE THE BACTERIAL CONTENT OF RAIN WATER AND SNOW

1. Collect a sample of rain water by allowing a wide-mouth, sterile bottle to stand open on a wood or metal surface where dust will not enter it.

2. Examine according to the procedure outlined in the previous experiment.

3. If snow is examined, collect a specimen aseptically in a sterile bottle and examine as outlined for water.

EXERCISE NO. 22: DETERMINATION OF THE NUMBER AND TYPES OF BACTERIA IN ICE

The methods for the examination of ice are identical with those used for water. The difficulty is to secure the specimen of ice and sample it without contamination.

1. Secure a large piece of the ice to be examined. This should preferably be a section of the entire original block, in order to have the core.

2. Wash the cake by pouring tap water, distilled water and finally sterile water over it.

3. By means of a sterile auger or bit, bore into the cake at the edge, allowing the first few chips to fall away. Then collect the ice shavings in a sterile wide-mouth bottle. Allow the shavings to melt, and analyze as described for water in Exercise No. 20.

4. Repeat the sampling with a second sterile bit at the center of the block, or core, which froze last.

EXERCISE NO. 23: ISOLATION OF THERMOPHILIC BACTERIA

1. Secure specimens of soil, sewage, milk, etc.

2. Inoculate tubes of melted agar medium and pour into sterile Petri dishes.

3. Incubate at 55° C., until growth has occurred.

4. Isolate pure cultures from isolated colonies and transfer to sterile agar slants.

5. Determine "Index Number" of one of the pure cultures; incubate all culture tubes at 55° C.

6. Make a series of broth and agar slant cultures and incubate at as many constant temperatures as possible. This will be determined by the number of incubators available in the laboratory.

All apparatus and culture media used for the propagation of thermophilic bacteria should be thoroughly sterilized (20–25 lbs. for sixty minutes) before washing.

EXERCISE NO. 24: MOLDS ON BREAD, FRUITS, ETC.

1. Secure a loaf of bread and store in a dark, moist place until molds have developed.

2. Isolate pure cultures of the molds which have developed.

3. Determine the species and characteristics.

EXERCISE NO. 25: BIOLOGICAL TEST FOR ARSENIC

1. Powder some bread and carefully mix the suspected materials thoroughly in it.

2. Transfer to flasks and sterilize in the Arnold or autoclav, after plugging.

3. When the flasks are cool, inoculate with *Penicillium brevicaule*. Incubate at 37° C.

4. After incubation, examine for a garlic-like odor of arsenic. This will be secured if arsenic was present in the suspected materials.

EXERCISE NO. 26: BACTERIOLOGICAL EXAMINATION OF DISH WATER

1. Collect a specimen of dish water in a sterile bottle.
2. Dilute this 1 : 100 and 1 : 1000.
3. Plate out in plain agar and incubate at 37° C.
4. Isolate pure cultures for identification or study, if desired.

EXERCISE NO. 27: TO DETERMINE THE TYPES OF BACTERIA IN MILK

1. Secure a sample of raw milk and a sample of pasteurized milk.

2. Prepare two agar plates from dilutions of 1 : 1000, 1 : 10,000 and 1 : 100,000. Incubate at 37° C. for forty-eight hours. *

3. Transfer all of the colonies from the plate for each sample which has the smallest number of bacteria, to tubes of sterile litmus milk. Incubate fourteen days at 37° C.

4. Group the bacteria, according to changes produced, into the following groups (Ayers and Johnson).

1. Acid-coagulating.
2. Acid-producing (without coagulation).
3. Inert—no change.
4. Alkali-forming.
5. Peptonizing.

EXERCISE NO. 28: TO STUDY THE INFLUENCE OF FREEZING ON BACTERIA

1. Prepare broth culture of *Escherichia coli* and *Bacillus subtilis*.

2. Sterilize some 2-c.c. ampoules in the autoclav. They may be placed upside down in a wire basket in the bottom of which is a layer of cotton.

* Instead of preparing a new set of plates, those prepared in Experiment No. 16, page 196, may be used.

3. After cultures have grown, fill ten ampoules with 1 c.c. of the broth cultures. This may require the use of a sterile syringe. Seal the filled ampoules in the flame of the Bunsen burner.

4. Count the numbers of cells in one of each set of ampoules.

5. Place the other eighteen in the ice trays of a so-called electric refrigerator.

6. Remove an ampoule every week and count the living cells.

7. Plot the results.

EXERCISE NO. 29: ISOLATION OF *RHIZOBIUM LEGUMINOSARUM* (*Bacillus radicicola*)

1. Secure some fresh roots of clover, alfalfa or other leguminous plants. Wash off the dirt very carefully.

2. Select one of the larger tubercles and place in a sterile test tube for five minutes with a little 0.5 per cent mercuric chloride.

3. Rinse the nodule with sterile water and transfer it to a sterilized slide.

4. Add a large drop of water and crush the tubercle.

5. Use a loopful of this suspension for inoculating a series of three melted ash maltose agar tubes and pour into Petri dishes.

6. Incubate at 20° C. After a few days, colonies of *Rhizobium leguminosarum* will appear as translucent, viscid drops.

7. Isolate and determine the characteristics.

EXERCISE NO. 30: TO STUDY THE TYPES OF CURD FORMED IN MILK

1. Inoculate tubes of sterile litmus milk with the following bacteria:

Bacterium coli.
Bacterium aerogenes.
Streptococcus lacticus.
Lactobacillus bulgaricus.

2. Incubate at 37° C. and examine for type of curd and gas formation.

EXERCISE NO. 31: STUDY OF BACTERIA IN THE MOUTH

1. Place about 1 c.c. of saliva in a tube of melted agar and pour into a Petri dish. Incubate at 37° C. for twenty-four hours.

2. Isolate pure cultures from the colonies and study, if desired.

3. Repeat the experiment with tartar scraped from the teeth.

EXPERIMENT NO. 32: EFFECT OF DYES (GENTIAN VIOLET AND MERCUROCHROME—220) ON BACTERIA [1]

1. Make divided plates by placing a strip of cardboard which has been dipped into paraffin across the center of Petri dishes; sterilize in the oven in the usual manner.

2. Pour melted cooled agar into one-half of the dish and allow it to solidify thoroughly.

3. By means of sterile forceps, remove the strip of cardboard and pour the dye agar described below into the other half of the dish until the surfaces of the agar are even. Allow this dye agar to harden. It is best to have the dye agar as cool as possible before pouring it into the dish in order to cause as little condensation as possible on the cover of the dish.

4. The following concentrations of dyes in the agar should be used: 1 : 1000, 1 : 20,000, 1 : 35,000, 1 : 50,000 and 1 : 100,000. The dyes may be made up in concentrations in water of 1 : 100, 1 : 1000, 1 : 3500, 1 : 5000 and 1 : 10,000. By adding 1 c.c. of these dilutions to 9 c.c. of sterile agar, the proper concentrations of dye agar will be secured.

5. These plates should then be streaked with Gram-negative and Gram-positive bacteria. *Escherichia coli* and *Bacillus subtilis* may be used.

EXERCISE NO. 33: STUDY OF BACTERIA IN AIR, DUST, ETC.

1. Pour three sterile Petri dishes with sterile plain agar. Allow to harden.

2. Carefully lift the cover of one dish and touch the tips of the fingers to the surface of the agar medium.

3. Inoculate the surface of the agar in the second dish by rubbing over it a piece of cotton which has been rubbed over a dusty floor.

4. The third dish should be inoculated by using a loopful of saliva. Spread this over the surface of the agar.

5. Incubate all plates at 37° C.

6. At the next period, number several of the isolated colonies on the bottom of the dish.

[1] This experiment requires a great amount of apparatus. It may be desirable to divide the class into groups of four or five and have each group carry out a part of the work. The results may then be brought together by the students in the group.

7. Make ordinary stains with the cells from several of these colonies until a rod-shaped organism and a coccus-shaped organism are isolated.

8. Make a Gram stain of these organisms and prepare agar slants for future experiments.

EXERCISE NO. 34: BACTERIA ON THE HANDS

1. Scrape the back of the hand with a sterile, dull knife blade or scalpel.

2. Transfer a little of the scrapings to a tube of sterile water. Shake thoroughly.

3. Plate out in plain agar.

4. Examine the colonies and isolate pure cultures for further study.

EXERCISE NO. 35: ISOLATION BACTERIA FROM BOILS OR PIMPLES

CAUTION.—These bacteria should be handled with great care since they are pathogenic.

1. Select a pimple or small boil and sterilize the adjacent surface with a little lipol or formaldehyde.

2. Open the boil or pimple, remove a little of the pus with a platinum needle and place in some sterile water.

3. Plate out in dextrose agar. Incubate at 37° C. for twenty-four or forty-eight hours.

4. Transfer a portion of an isolated colony to a sterile agar slant.

5. Study the pure culture according to the routine procedures for pure cultures.

EXERCISE NO. 36: BACTERIA ON COINS AND THE OLIGODYNAMIC ACTION OF METALS

1. Take a coin and drop it into a sterile tube or flask containing 10 c.c. of water with a little sand. Shake thoroughly to remove the bacteria from the coin.

2. Plate out 1 c.c. of the wash water in plain agar.

3. Take another coin and scour with soap and brush. Drop into 60 per cent alcohol or boiling water for five minutes.

4. With sterile forceps, place the coin in the center of a sterile Petri dish and pour over the coin a tube of agar which has been inoculated with *Serratia marcescens.*

5. Incubate at 37° C. for twenty-four to forty-eight hours.
6. Note the type of growth which occurs about the coin.

EXERCISE NO. 37: EXAMINATION OF CANDY

1. Purchase some candy on the open market and dissolve in sterile water. (If quantitative data are desired, the candy must, of course, be weighed and dissolved in a known amount of water.)
2. Plate out in plain agar and incubate plates at 37° C. for twenty-four hours.
3. Isolate and determine characteristics of the organisms, if desired.
4. Repeat the experiment with cinnamon and peppermint drops.

EXERCISE NO. 38: THE CUP PLATE METHOD FOR DETERMINING DISINFECTING VALUE

Melt 20 c.c. of plain nutrient agar, cool to 45° C., add 4 c.c. of sterile blood serum;* then add one drop of a 24-hour broth culture of *Staphylococcus aureus*, mix thoroughly by pouring back and forth into another sterile culture tube. After mixing, pour into a plate into the center of which a sterile glass stopper, 2 cm. in diameter, has been placed, and allow to harden. After the serum-agar has cooled and hardened, remove the stopper (a glass vial may also be used) by slightly twisting; seal any small cracks and crevices which may be formed with two or three drops of sterile melted, cooled agar. The antiseptic to be tested is then placed in the cup, being enough to almost fill it. Reddish added 0.25 c.c. of antiseptic to the cup when 20 c.c. of agar are used. Incubate with an unglazed clay top at 37° C., for 24 and 48 hours. If the antiseptic possesses penetrating powers and is not counteracted by the serum, a clear zone will surround the cup. The width of this zone can then be measured from the edge of the cup to the edge of the zone. To differentiate between actual germicidal power or bacteriostatic activity, small pieces of agar with colonies may be picked from the clear zone and subcultures made in plain broth. A control plate may also be made without serum.

* If it is impossible to secure blood serum in sufficient amounts for the needs of a large class, the experiment may be made without it. It is possible that diluted egg-white might be substituted.

NOTES

NOTES

NOTES

NOTES

APPENDIX

Preparation of Reagents

The reagents required by the bacteriologist are in many cases those used in the chemistry laboratory. Many chemical methods are used for studying bacteria, and those trained in chemistry, especially biochemistry, are best able to understand the changes brought about by bacteria.

The reagents described below are those which are useful in introductory courses.

Nomal Sodium Hydroxide.—Weigh out 40.06, or better about 45 grams, of sodium hydroxide and dissolve in 1 liter of boiled, cooled, distilled water. Standiardize against normal hydrochloric acid. It is best to allow the two solutions to stand at the same temperature for several hours or over night. The titration should be repeated several times. Calculation is as follows:

If

$$50 \text{ c.c. NaOH} = 52.6 \text{ N HCl}$$

the NaOH is too strong by 2.6 c.c. per 50 c.c.

$$50 : 2.6 = 1000 : X \quad X = \frac{2.6 \times 1000}{50} = 50 \text{ c.c.}$$

This amount of water should be added, after which the solution will be exactly normal. It should be checked, however, against the N HCl. From this the N/20 NaOH may be prepared by diluting 50 c.c. of the N NaOH to a liter.

Normal Hydrochloric Acid.—This should contain 36.46 grams of HCl in a liter of water. The pure concentrated HCl may be diluted to a specific gravity of 1.020. This solution is too strong, having about 4.13 grams of HCl in a liter. To be made exactly normal, it should be titrated against pure Na_2CO_3. This is obtained by heating the $NaHCO_3$, being careful not to allow it to fuse. This is then put into a weighing bottle from which it is weighed into Erlenmeyer flasks for titrating. Dissolve in boiled distilled-water and titrate with the HCl solution, using methyl orange as the indicator. Calculations are made as follows, assuming, for example, that 1.9864 grams of Na_2CO_3 neutralized 37.28 c.c. of the HCl solution.

If our solution had been exactly normal, 1 liter would have neutralized

$$\frac{Na_2CO_3}{2} = \frac{106.00}{2} \quad 53 \text{ grams of } Na_2CO_3$$

$$53 : 1000 = 1.9864 : X$$

$$X = 37.48.$$

37.28 c.c. of the HCl solution were required, and it is evident that this solution is too strong and that for each 37.28 c.c. of the solution 37.48 − 37.28 = .20 c.c. of water must be added. The amount of water to be added to each liter of the HCl solution to make it exactly normal is computed as follows:

$$37.28 : .20 = 1000 : X$$

X = amount of distilled water to be added.

Preparation of Staining Solutions

The following formulae were taken from the Coleman & Bell Company, Inc., catalog. In laboratories where small amounts of staining solutions are used, it may be more convenient to purchase the prepared solutions.

Methylene Blue—Saturated Aqueous Solution.

Weigh out
Methylene blue (for bacilli) ..... 1.5 grams
Place in a stoppered bottle having a capacity of 150 to 200 c.c. and add
Distilled water ..... 100.0 c.c.
Allow the water to remain in contact with the dye for two days with occasional shaking. Filter.

Methylene Blue—Saturated Alcoholic Solution.

Weigh out
Methylene blue (for bacilli) ..... 1.5 grams
Place in a stoppered bottle of 150 c.c. capacity and add
Ethyl alcohol ..... 100.0 c.c.
Allow the alcohol to remain in contact with the dye for two hours, shaking vigorously every few minutes. Filter.

Loeffler's Methylene Blue.

Dissolve 1.0 gram potassium hydroxide in 100.0 c.c. of distilled water. This makes a 1 : 100 stock solution of potassium hydroxide. Take 1.0 c.c. of the stock solution and add 99.0 c.c. distilled water. This makes a 1 : 10,000 KOH solution. Add to the latter methylene blue—saturated alcoholic solution ..... 30.0 c.c.
Shake and filter.

Gentian Violet—Saturated Aqueous Solution.

Weigh out
Gentian violet improved ..... 2.25 grams
and proceed as in preparing the corresponding solution of methylene blue.

Gentian Violet—Stabilized (Stovall and Nichols, 1916).

To prevent the usual deterioration of gentian violet, the following stain is proposed:

Aniline	28.0	c.c.
Gentian violet	8.0	grams
95 per cent alcohol	100.0	c.c.
Normal hydrochloric acid	5.0	c.c.
Distilled water	100.0	c.c.

The gentian violet should be dissolved in the alcohol. The aniline and hydrochloric acid may be mixed and diluted to 900.0 c.c. Filter these solutions and add to each other with a subsequent filtration.

Gentian Violet—Stabilized (Kilduffe, 1909).

Another formula for gentian violet for use in the Gram stain has been given by Kilduffe (1909). This has been used in the author's laboratory and has given good results.

Solvent

5 c.c. commercial formalin.
95 c.c. distilled water.

Stain

25 c.c. saturated alcoholic gentian violet.
75 c.c. of the solvent.
or
16 c.c. saturated gentian violet.
84 c.c. solvent.

Gentian Violet—Saturated Alcoholic Solution.

Weigh out

Gentian violet improved	5.0	grams

and proceed as in preparing the corresponding solution of methlene blue.

Carbol Gentian Violet.

Measure out and mix

Gentian violet—saturated alcoholic solution	10.0	c.c.
Carbolic acid, 1.0 per cent aqueous solution	100.0	c.c.

Filter.

Aniline Water Gentian Violet.

Measure out

Distilled water	100.0	c.c.

Add 0.5 c.c. aniline and shake. Filter, and add Gentian violet—saturated alcoholic solution 100.0 c.c.

Filter.

NOTE: This solution will not keep longer than fourteen days.

Fuchsin Basic—Saturated Aqueous Solution.

Weigh out
Basic fuchsin-pure crystals..... 1.5 grams
and proceed as in preparing the corresponding solution of methylene blue.

Fuchsin Basic—Saturated Alcoholic Solution.

Weigh out
Basic fuchsin—pure crystals..... 5.0 grams
and proceed as in preparing the corresponding solution of methylene blue.

Carbol Fuchsin.

Measure and mix
Basic fuchsin—saturated alcoholic solution..... 10.0 c.c
Carbolic acid—3.0 per cent aqueous solution..... 90.0 c.c
Filter.

Eosin-aqueous Solution.

Weigh out
Eosin Y..... 1.0 gram
and dissolve in
Distilled water..... 100.0 c.c.
Filter.

Eosin-alcoholic Solution.

Weigh out
Eosin Y..... 0.5 gram
and dissolve in
Alcohol (70 per cent)..... 100.0 c.c.
Filter.

Safranine-Aqueous Solution.

Weigh out
Safranine Y..... 1.0 gram
and dissolve in
Distilled water..... 100.0 c.c.
Filter.

Neutral Red—Aqueous Solution.

Weigh out
Neutral red..... 1.0 gram
and dissolve in
Distilled water..... 100.0 c.c.
Filter.

Bismarck Brown—Aqueous Solution.

Weigh out
Bismarck brown ..... 0.5 gram
and dissolve in
Distilled water ..... 100.0 c.c.
Filter.

Haematoxylin.

Dissolve 1.0 gram haematoxylin, white crystals, C.P. in 10.0 c.c. alcohol.
Weigh out
Ammonium alum ..... 2.0 grams
and dissolve in
Distilled water ..... 100.0 c.c.
Bring the alum solution to a boil and add alcoholic solution of haematoxylin. Then add
Mercuric oxide ..... 0 5 gram
and allow to act for two minutes. Filter and allow to cool. Add
Glycerine ..... 25 0 c.c.
Glacial acetic acid ..... 10.0 c.c.
Shake until thoroughly mixed.

Lugol's Iodine Solution (Gram's).

Weigh out
Iodine ..... 1 0 gram
Potassium iodide ..... 3.0 gram
Dissolve in 25.0 c.c. distilled water by heating. Add solution to 275.0 c.c. distilled water. Shake and filter.

Goré Test for Indol; Reagents:

Solution 1.
Para-dimethyl-amido-benzaldehyde ..... 1 0 gram
Absolute alcohol ..... 95 0 c.c.
Hydrochloric acid ..... 20.0 c.c.

Solution 2.
Potassium persulfate ..... 1 0 gram[1]
Distilled water ..... 100.0 c.c.

[1] Some who are using this test employ a saturated solution of persulfate.

METRIC EQULVALENTS

Linear Measure

1 centimeter = 0.3937 inch
1 decimeter = 3.937 inches = 0.328 foot
1 meter = 39.37 inches = 1.0936 yards
1 dekameter = 1.9884 rods
1 kilometer = 0.62137 mile
1 inch = 2.54 centimeters
1 foot = 3.048 decimeters
1 yard = 0.9144 meter
1 rod = 0.5029 dekameter
1 mile = 1.6093 kilometers

Square Measure

1 square centimeter = 0.1550 square inch
1 square decimeter = 0.1076 square foot
1 square meter = 1.96 square yards
1 are = 3.954 square rods
1 hektar = 2.47 acres
1 square kilometer = 0.386 square mile
1 square inch = 6.452 square centimeters
1 square foot = 9.2903 square decimeters
1 square yard = 0.8361 square meter
1 square rod = 0.2529 are
1 acre = 0.4047 hektar
1 square mile = 2.59 square kilometers

Measure of Volume

1 cubic centimeter = 0.061 cubic inch
1 cubic decimeter = 0.0353 cubic feet
1 cubic meter, 1 stere = 1.308 cubic yards; 0.2759 cord
1 liter = 0.908 quart dry; 1.0567 quart liquid
1 dekaliter = 2.6417 gallons; 0.135 peck
1 hektoliter = 2.8375 bushels
1 cubic inch = 16.39 cubic centimeters
1 cubic foot = 28.317 cubic decimeters
1 cubic yard = 0.7646 cubic meter
1 cord = 3.624 steres
1 quart dry = 1.101 liters
1 quart liquid = 0.9463 liter
1 gallon = 0.3785 dekaliter
1 peck = 0.881 dekaliter
1 bushel = 0.3524 hektoliter

Weights

1 gram	= 0.0527 ounce
1 kilogram	= 2.2046 pounds
1 metric ton	= 1.1023 English ton
1 ounce	= 28.35 grams
1 pound	= 0.4536 kilogram
1 English ton	= 0.9072 metric ton

Approximate Metric Equivalents

1 decimeter	= 4 inches
1 meter	= 1.1 yards
1 kilometer	= $\frac{5}{8}$ of mile
1 hektar	= $2\frac{1}{2}$ acres
1 stere, or cubic meter	= $\frac{1}{4}$ of a cord
1 liter	= 1.06 quart liquid = 0.9 quart dry
1 hektoliter	= $2\frac{5}{8}$ bushels
1 kilogram	= $2\frac{1}{6}$ pounds
1 metric ton	= 2200 pounds

Preparation of Indicator Solutions

Azolitmin Solution.—Add 1 per cent of Kahlbaum's azolitmin to distilled water and boil for 5 minutes. Adjust the reaction, if necessary, with dilute sodium hydroxide. When used in a medium it should give a distinct blue medium.

Andrade's Indicator.

0.5 per cent aqueous solution of acid fuchsin	100.0 c.c.
Normal sodium hydroxide	16.0 c.c.

This indicator is added to media in concentrations of about 1 per cent.

Indicator Solutions for the Determination of Hydrogen-ion Concentrations

Solutions for the La Motte Comparator Sets.—The indicator solutions used with the La Motte Block and Roulette Comparators are made by dissolving the dry indicators in sodium hydroxide solution and then making up to the required volume with distilled water.

Table VIII gives the data required for preparation of these solutions. The first column gives the name of the indicator, the second the strength of the finished solution for the various indicators, the third the weight of dry indicator required to give 1 liter of indicator solution of the strength indicated in the second column, the fourth the number of cubic centimeters of M/20 sodium hydroxide required to dissolve the weight of dry indicator given in the third column, the fifth the color which the solution should have when completed.

TABLE VIII

Indicator	Strength	Weight of Dye for 1 Liter, Grams	M/20 NaOH Initially Used, Cubic Centimeters	Color of Neutral Solution of Dye
Meta-cresol purple.....	0.04	0.4	23.3	Yellow
Brom-phenol blue.....	0.04	0.4	16.5	Blue
Brom-cresol green.....	0.04	0.4	12.8	Blue
Brom-cresol purple.....	0.04	0.4	16.3	Purple
Chlorphenol red.....	0.04	0.4	20.7	Red
Brom-thymol blue.....	0.04	0.4	14.1	Green
Phenol red.....	0.02	0.2	12.5	Orange
Cresol red.....	0.02	0.2	11.7	Yellow
Thymol blue.....	0.04	0.4	18.9	Yellow

All the indicator solutions, *with the exception of brom-cresol purple*, are prepared by grinding the weighed amount of dye in a mortar with the given volume of sodium hydroxide solution until the maximum of dye has dissolved. The mixture is then transferred to a liter beaker, the mortar being completely washed with distilled water. Distilled water is then added until a volume of about 250 c.c. is reached. The contents of the beaker are then heated over a burner or hot plate at 80–90° C. and stirred frequently until solution of the dye is complete. In the case of certain dyes it may be necessary to add additional sodium hydroxide to dissolve all the dye. On cooling, the indicator solution is transferred quantitatively to a calibrated liter flask, diluted to the mark with distilled water and thoroughly mixed. The indicator solution is now ready for use.

A practical method of determining just when sufficient sodium hydroxide has been added is to be sure that all the dye has dissolved and that the solution, when viewed in a thin layer, as in the stem of a pipette, has the color of a neutral solution, as indicated in column five. The *least* amount of sodium hydroxide necessary to give the neutral color, and at the same time dissolve all the dye, is the proper amount. Final adjustment of the solution should be made on the diluted solution, care being taken that the desired volume is not exceeded.

Due to the instability of brom-cresol purple in strong alkali, *a different procedure must be followed.* The weighed amount of dye is placed in a liter beaker and 750–800 c.c. of water added. While stirring this mixture thoroughly, the sodium hydroxide solution is run in slowly. The preparation is then completed, following the method given for the other dyes.

Solutions for the La Motte Hydrogen-ion Testing Sets.—Indicator solutions for the La Motte Hydrogen-ion Testing Sets are prepared by dissolving the weighed amount of the dye in ethyl alcohol, adding the required volume of sodium hydroxide solution and diluting with additional alcohol. The solution should contain at least 60 per cent alcohol. No denaturant that is insolu-

ble in water, as benzene or gasoline, or that will impart undue acidity or alkalinity, should be present.

Solutions of methyl red are best prepared by dissolving 0.2 gram of the dye in about 200 c.c. of ethyl alcohol to which 14.8 c.c. of M20 sodium hydroxide solution has been added, and warming gently. When solution is complete, cool and make up to 1 liter with alcohol.

The following are approximate equivalents:

EQUIVALENTS OF GRAMS OR CUBIC CENTIMETERS IN APOTHECARY'S WEIGHT

(From Merck's Materia Medica)

0.001	gram	or	c.c.	=	$\frac{1}{60}$	grn.	or	min.	0.3	gram	or	c.c. = 5 grn. or min.
0.003	"	"	"	=	$\frac{1}{20}$	"		"	0.5	"	"	= 8 " "
0.004	"	"	"	=	$\frac{1}{15}$	"		"	0.6	"	"	= 10 " "
0.008	"	"	"	=	$\frac{1}{8}$	"		"	0.8	"	"	= 12 " "
0.01	"	"	"	=	$\frac{1}{6}$	"		"	1	"	"	= 15 " "
0.015	"	"	"	=	$\frac{1}{4}$	"		"	4	"	"	= 1 dr. or fl-dr.
0.03	"	"	"	=	$\frac{1}{2}$	"		"	15	"	"	= 4 " "
0.05	"	"	"	=	$\frac{3}{4}$	"		"	30	"	"	= 1 oz. or fl-oz.
0.6	"	"	"	= 1		"		"	120	"	"	= 4 " "
0.1	"	"	"	= $1\frac{1}{2}$		"		"	237	"	"	= 8 " "
0.2	"	"	"	= 3		"		"	475	"	"	= 1 lb. or pint
0.25	"	"	"	= 4		"		"	950	"	"	= 2 " "

Indicators for the Determination of Hydrogen-ion Concentration.—The column showing the *p*H range has been compiled from the works of Sorensen, Clark and Lubs and other writers. (By permission of the British Drug House, Ltd., of London, Eng.)

	Useful Range K as *p*H	Color Change
Alarzin	11.0–13.0	pink to violet
Alizarin blue S	9.0–11.0	brown to green
	11.0–13.0	green to violet
Alizarin red S	4.0–6.0	yellow to orange-red
	6.0–12.0	orange to red-violet
Alizarin yellow G. *p*-nitrobenzene-azo-salicylic acid	10.1–12.1	yellow to red-orange
Alizarin yellow G. G. *m*-nitrobenzene-azo-salicylic acid	10.0–12.0	colorless to yellow
Alkali blue 5 B	9.4–14.0	blue to red-violet
Azolitmin	5.0–8.0	red to blue

	Useful Range K as *p*H.	Color Change
Benzene-azo-benzylaniline	0.5– 2.0	red to yellow
Benzene-azo-α-naphthylamine	3.7– 5.0	red to orange
Benzyl-aniline-azo-benzene sulfonic acid	1.9– 3.3	orange-red to yellow
Brilliant cresyl blue	– 0.2– 1.0	red-orange to blue
	12.0–12.4	blue to yellow
Brilliant yellow	6.4– 9.4	yellow to red-orange
Bromo-cresol green	3.6– 5.2	yellow to blue
Bromo-cresol purple	5.2– 6.8	yellow to violet
Bromo-phenol blue	2.8– 4.6	yellow to violet
Bromo-thymol blue	6.0– 7.6	yellow to blue
Chloro-phenol red	4.6– 7.0	yellow to violet-red
Cochineal (tincture)	5.0– 6.0	yellow to purple
Congo red	3.0– 5.0	violet to red-orange
***o*-Cresol-phthalein**	8.2– 9.8	colorless to violet-red
***m*-Cresol purple**	0.5– 2.5	red to yellow
	7.6– 9.2	yellow to violet
Cresol red	7.2– 8.8	yellow to violet-red
Cresyl fast violet	2.0–12.4	violet to yellow
Diethyl red	4.5– 6.5	red to yellow
Dimethyl-amino-azo-benzene	2.9– 4.0	red to yellow
Ethyl orange	3.0– 4.5	red to orange
Haematoxylin	0 – 1.0	pink to green
	6.0–11.0	orange to red-violet
Iodo-eosin	.....	.....
Lacmoid	4.0– 6.0	orange-red to violet
Litmus (aqueous)	5.0– 8.0	red to blue
Litmus (Kubel-Tieman's)	5.0– 8.0	red to blue
Litmus (tincture)	5.0– 8.0	red to blue
Martius yellow	2.0– 3.2	colorless to yellow
Metanil yellow	1.2– 2.3	red to yellow
Methyl orange	2.9– 4.0	orange-red to orange-yellow
Methyl orange (improved) Moir's	3.0– 5.0	orange to blue-violet
Methyl red	4.2– 6.3	red to yellow
Methyl red (water soluble)	4.2– 6.3	red to yellow

	Useful Range K as *p*H.	Color Change
Methyl red phenolphthalein	4.2–10.0	red to yellow to red
Methyl thymol blue	4.0–10.0	red to yellow to blue
Methyl violet	0.1– 3.2	yellow to violet
α-Napththol-benzein	8.5– 9.8	yellow to green
α-Naphthol-phthalein	7.3– 8.7	yellow to blue
Naphthyl red	3.2– 4.6	red to pale orange
Naphthylamine-azo-benzene-*p*-sulfonic acid	4.2– 5.8	pink to orange
Neutral red	6.8– 8.0	red to orange
***p*-Nitrophenol**	5.0– 7.0	colorless to yellow
Para-methyl red *p*-carboxy-benzene-azo-dimethylaniline	1.6– 3.2	red to orange
Phenacetolin	1.0–13.0	yellow to red
Phenolphthalein	8.3–10 0	colorless to violet-red
Phenol red	6.8 8.4	yellow to red
Phenol-tetrachloro-phthalein	8.2– 9.4	colorless to pink
Phenol thymol phthalein	8.3–11 0	colorless to red to violet
Phenol violet	8 0–10.0	yellow to blue to violet
Phenyl-α-naphthylamine-azo-benzene-*p*-sulfonic acid	3.0– 5 0	violet to red-orange
Phenyl-α-naphthylamine-azo-*o*-carboxy-benzene	5 0– 7.0	violet to yellow
Porrier's blue	11.0–13.0	blue to red-violet
Resazurine	5.0– 7.0	pink to violet
Rosolic acid	6.9– 8.0	orange to red
Sulfo-naphthyl red	5.2– 6.8	red to yellow
Thymol blue	1.2– 2.8	red to yellow
	8.0– 9.6	yellow to blue
Thymol phthalein	9.3–10.5	colorless to blue
Thymol violet	9.0–13.0	yellow to green to violet
Titan yellow (Clayton yellow)	12.0–13.0	yellow to red
Tropaeolin O (resorcin yellow)	11.1–12.7	yellow to orange
Tropaeolin OO (orange IV)	1.4– 2.6	pink to yellow
Tropaeolin OOO (orange II)	7.0– 8.9	yellow to red
Tropaeolin D (*vide* methyl orange)	.....	.....

THERMOMETERS

$$\text{Fahrenheit}^\circ = \frac{\text{Centigrade}^\circ \times 9}{5} + 32. \qquad \text{Centigrade}^\circ = \frac{\text{Fahrenheit}^\circ - 32 \times 5}{9}$$

Ready Reference Comparison of Centigrade and Fahrenheit Thermometer Scales

Cent. °	Fah. °	Cent. °	Fah. °	Cent. °	Fah. °	Cent. °	Fah. °	Cent. °	Fah. °	Cent. °	Fah. °
−40	−40	7	44.6	38	100.4	69	156.2	99	210.2	245	473
−35	−31	8	46.4	39	102.2	70	158.0	100	212.0	250	482
−30	−22	9	48.2	40	104.0	71	159.8	105	221.0	255	491
−25	−13	10	50.0	41	105.8	72	161.6	110	230.0	260	500
−20	− 4.0	11	51.8	42	107.6	73	163.4	115	239.0	265	509
−19	− 2.2	12	53.6	43	109.4	74	165.2	120	248.0	270	518
−18	− 0.4	13	55.4	44	111.2	75	167.0	125	257.0	275	527
−17	1.4	14	57.2	45	113.0	76	168.8	130	266.0	280	536
−16	3.2	15	59.0	46	114.8	77	170.6	135	275.0	285	545
−15	5.0	16	60.8	47	116.6	78	172.4	140	284.0	290	554
−14	6.8	17	62.6	48	118.4	79	174.2	145	293.0	295	563
−13	8.6	18	64.4	49	120.2	80	176.0	150	302.0	300	572
−12	10.4	19	66.2	50	122.0	81	177.8	155	311.0	310	590
−11	12.2	20	68.0	51	123.8	82	179.6	160	320.0	320	608
−10	14.0	21	69.8	52	125.6	83	181.4	165	329.0	330	626
− 9	15.8	22	71.6	53	127.4	84	183.2	170	338.0	340	644
− 8	17.6	23	73 4	54	129.2	85	185 0	175	347.0	350	662
− 7	19.4	24	75.2	55	131 0	86	186 8	180	356.0	360	680
− 6	21.2	25	77.0	56	132.8	87	188 6	185	365.0	370	698
− 5	23 0	26	78 8	57	134.6	88	190 4	190	374.0	380	716
− 4	24.8	27	80.6	58	136.4	89	192 2	195	383.0	390	734
− 3	26.6	28	82.4	59	138.2	90	194 0	200	392.0	400	752
− 2	28.4	29	84.2	60	140.0	91	195 8	205	401.0	420	788
− 1	30.2	30	86.0	61	141.8	92	197.6	210	410.0	440	824
0	32.0	31	87.8	62	143.6	93	199.4	215	419.0	460	860
1	33.8	32	89.6	63	145.4	94	201.2	220	428.0	480	896
2	35.6	33	91.4	64	147 2	95	203.0	225	437.0	500	932
3	37.4	34	93.2	65	149 0	96	204.8	230	446.0	520	968
4	39.2	35	95 0	66	150.8	97	206.6	235	455.0	540	1004
5	41.0	36	96.8	67	152.6	98	208.4	240	464.0	560	1040
6	42.8	37	98.6	68	154 4						

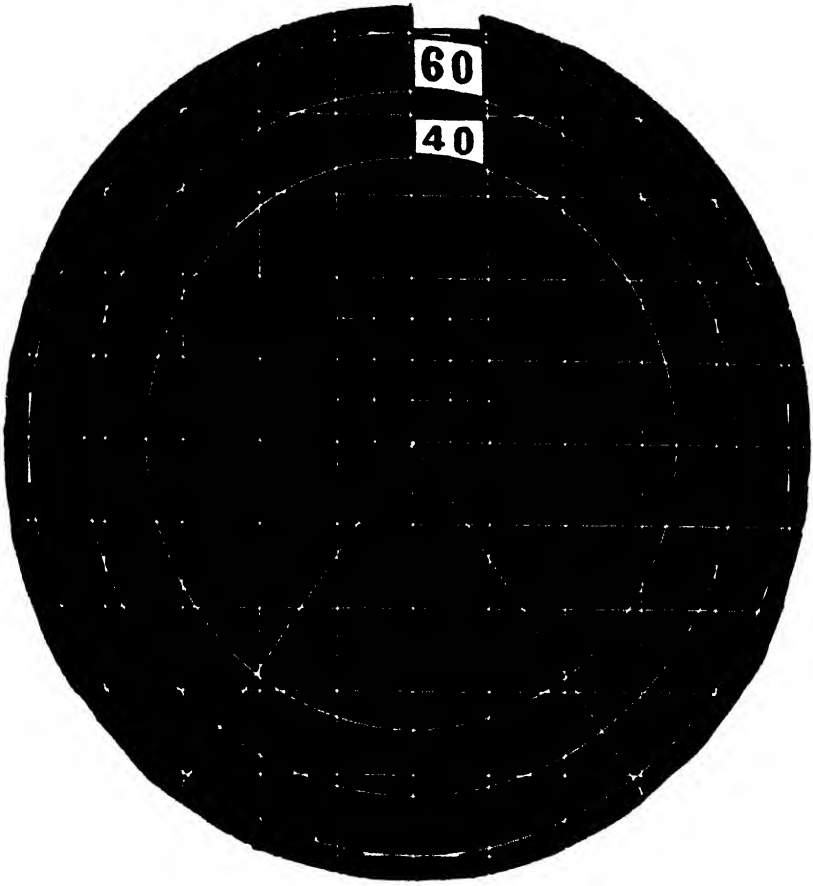


FIG. *B*.—Frost's Plate Counter.

Each of the larger squares represents an area of 1 sq. cm. The figures 60 and 40 indicate the number of square centimeters in the circles bounded by the respective lines. Each sector is one-tenth the area of its circle.

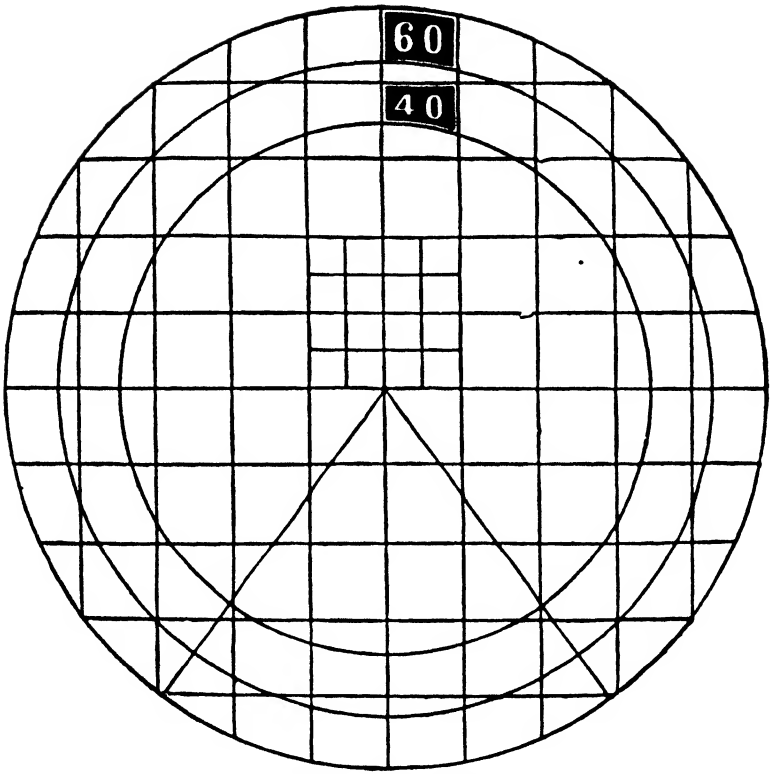


FIG. C.—Frost's Plate Counter.

Each of the larger squares represents an area of 1 sq. cm. The figures 60 and 40 indicate the number of square centimeters in the circles bounded by the respective lines. Each sector is one-tenth the area of its circle.

REFERENCES

Textbooks on Laboratory Technic

Conn, H. J. An Elementary Laboratory Guide in General Bacteriology The William & Wilkins Co., Baltimore, Md., 1927.

Cunningham, A. Practical Bacteriology. An Introductory Course for Students of Agriculture. Oliver & Boyd, Edinburgh, 1924.

Ellis, D. Practical Bacteriology for Chemical Students. Longmans, Green & Co., London, 1923.

Fred, E. B. A Laboratory Manual of Soil Bacteriology. W. B. Saunders & Co., Philadelphia, 1916.

Frost, W. D. A Laboratory Guide in Elementary Bacteriology. The Macmillan Co., New York, 1918.

Giltner, W. Laboratory Manual in General Microbiology. John Wiley & Sons, Inc., New York, 1926.

Levine, Max. An Introduction to Laboratory Technique in Bacteriology. The Macmillan Co., New York, 1927.

Löhnis, F., Stevenson, W., and Smith, J. H. Laboratory Methods in Agricultural Bacteriology. Chas. Griffin & Co., Ltd., London, 1913.

Mackie, T. J., and McCartney, J. E. An Introduction to Practical Bacteriology as Applied to Medicine and Public Health. E. & S. Livingstone, Edinburgh, 1925.

Moore, V. A. Laboratory Exercises for Beginners in Bacteriology. Ginn & Company, New York, 1905.

Moore, V. A., and Hagan, W. A. Laboratory Manual in General and Pathogenic Bacteriology and Immunity. Ginn & Co., New York, 1925.

Norton, J. F., and Falk, I. S. Laboratory Outlines in Bacteriology and Immunology. The University of Chicago Press, Chicago, 1926.

Novy, F. G. Laboratory Work in Bacteriology. Geo. Wahr, Ann Arbor, Michigan, 1899.

Reed, H. S. A Manual of Bacteriology. Ginn & Co., New York, 1914.

Wadsworth, A. B. Standard Methods of the Division of Laboratories and Research of the New York State Department of Health. The Williams & Wilkins Co., Baltimore, Md.

Young, C. C. The Development, Organization and Operation of the Bureau of Laboratories. Robert Smith Co. (State Printers), Lansing, Mich.

Textbooks

Abbott, A. C. The Principles of Bacteriology: A Practical Manual for Students and Physicians. Lea & Febiger, Philadelphia, 1921.

Allen, P. W. Industrial Fermentations. The Chemical Catalogue Co., New York, 1926.

Bailey, E. H. S. Sanitary and Applied Chemistry. The Macmillan Co., New York, 1917.

Ball, M. V. Essentials of Bacteriology. W. B. Saunders & Co., Philadelphia, 1913.

Besson, A. Practical Bacteriology, Microbiology, and Serum Therapy. Longmans, Green & Co., New York.

Bigger, J. W. Handbook of Bacteriology for Students and Practitioners of Medicine. William Wood & Co., New York, 1925.

Broadhurst, J. How We Resist Disease: An Introduction to Immunity. J. B. Lippincott Co., Philadelphia, 1923.

Broadhurst, J. Bacteria in Relation to Man: A Study Text in General Microbiology. J. B. Lippincott Co., Philadelphia, 1925.

Broadhurst, J. Home and Community Hygiene. J. B. Lippincott Co., Philadelphia.

Buchanan, R. E., and Buchanan, E. D. Bacteriology. The Macmillan Co., New York.

Buchanan, R. E. Agricultural and Industrial Bacteriology. D. Appleton & Co., New York, 1921.

Buchanan, R. E. General Systematic Bacteriology: History, Nomenclature, Groups of Bacteria. Waverly Press, Baltimore, 1925.

Chandler, A. C. Animal Parasites and Human Disease. John Wiley & Sons, Inc., New York, 1926.

Conn, H. W., and Conn, H. J. Bacteriology. Waverly Press, Baltimore, 1926.

Conn, H. W. Agricultural Bacteriology. P. Blakiston's Son & Co., Philadelphia.

Conn, H. W. Bacteria, Yeasts and Molds in the Home.

Dakin, H. D., and Dunham, E. K. A Handbook on Antiseptics. The Macmillan Co., New York, 1917.

Dieudonné, A. (Translated by Bolduan, C. F.) Bacterial Food Poisoning. E. B. Treat & Co., New York, 1909.

Dukes, C. The Bacteriology of Food. H. K. Lewis & Co., London, 1925.

Eisenberg, A. A. Principles of Bacteriology. C. V. Mosby Co., St. Louis, 1920.

Ellis, D. Outlines of Bacteriology. Longmans, Green & Co., New York, 1909.

FISCHER, A. Structure and Functions of Bacteria. (Trans. by A. C. Jones.) Oxford, At the Clarendon Press, 1900.

FOWLER, G. J. Bacteriological and Enzyme Chemistry. E. Arnold, London, 1911.

FROST, W. D., and MCCAMPBELL, E. F. A Text Book of General Bacteriology.

FRANKLAND, MRS. PERCY. Bacteria in Daily Life. Longmans, Green & Co., New York, 1903.

GILTNER, W. Laboratory Manual in General Microbiology. John Wiley & Sons, Inc., New York, 1926.

GREAVES, J. E. Agricultural Bacteriology. Lea & Febiger, Philadelphia, 1922.

GREAVES, J. E., and GREAVES, E. O. Bacteria in Relation to Soil Fertility. D. Van Nostrand Co., New York, 1925.

GREAVES, J. E., and GREAVES, E. O., Elementary Bacteriology. W. B. Saunders Co., Phila., 1928.

GUILLIERMOND, A. The Yeasts. Translated by F. W. Tanner. John Wiley & Sons, Inc., New York, 1920.

HEINEMANN, P. Milk. W. B. Saunders & Co., Philadelphia, 1921.

HEWLETT, R. T. A Manual of Bacteriology. J. & A. Churchill, London, 1921.

HOLMAN, R. M., and ROBBINS, W. W. A Textbook of General Botany for Colleges and Universities. John Wiley & Sons, Inc., New York, 1927.

HORWOOD, M. P. Public Health Surveys. John Wiley & Sons, Inc., New York, 1921.

JORDAN, E. O. A Textbook of General Bacteriology. W. B. Saunders & Co., Philadelphia, 1921.

JORDAN, E. O. Food Poisoning. University of Chicago Press, Chicago.

KENDALL, A. I. Bacteriology, General, Pathological, and Intestinal. Lea & Febiger, Philadelphia.

KLEIN, L. A. Principles and Practice of Milk Hygiene. J. B. Lippincott Co., Philadelphia, 1917.

KINNICUTT, L. P., WINSLOW, C.-E. A., and PRATT, R. W. Sewage Disposal. John Wiley & Sons, Inc., New York, 1919.

KOLMER, J. A. Infection, Immunity, and Specific Therapy. W. B. Saunders & Co., Philadelphia.

LAFAR, F. Technical Mycology. The Utilization of Microorganisms in the Arts and Manufactures. Chas. Griffin & Co., Ltd., London, 1910.

LANE-CLAYTON, J. Milk and Its Hygienic Relations. Longmans, Green & Co., London, 1916.

LEVINE, M. An Introduction to Laboratory Technique in Bacteriology. The Macmillan Co., New York.

LIPMAN, J. G. Bacteriology in Relation to Country Life.

LÖHNIS, F., and FRED, E. B. Textbook of Agricultural Bacteriology. McGraw-Hill Book Co., Inc., New York, 1923.

LEDINGHAM, J. C. G. The Carrier Problem in Infectious Diseases. Longmans, Green & Co., New York, 1912.

MCFARLAND, J. A Textbook upon the Pathogenic Bacteria and Protozoa for Students of Medicine and Physicians. W. B. Saunders & Co., Philadelphia, 1919.

MACNEAL, W. J. Pathogenic Microorganisms: A Textbook of Microbiology for Physicans and Students of Medicine. P. Blakiston's Son & Co., Philadelphia.

MARSHALL, C. E. Microbiology. P. Blakiston's Son & Co., Philadelphia.

MASON, W. P. Examination of Water. John Wiley & Sons, Inc., New York, 1917.

MOORE, V. A. Principles of Microbiology. The Macmillan Co., New York, 1916.

MORREY, C. B. Fundamentals of Bacteriology. Lea & Febiger, Philadelphia, 1921.

MUIR, R., and RITCHIE, J. Manual of Bacteriology. Henry Frowde and Hodder & Stoughton, London, 1919.

NEWMAN, GEO., Bacteriology and the Public Health. P. Blakiston's Son & Co., Philadelphia.

ORLA-JENSEN, S. Dairy Bacteriology. P. Blakiston's Son & Co., Philadelphia, 1921.

PARK, W. H. Public Health and Hygiene. Lea & Febiger, Philadelphia, 1920.

PARK, W. H., and WILLIAMS, A. W. Pathogenic Microorganisms. Lea & Febiger, Philadelphia.

PARKER, H. N. City Milk Supply. McGraw-Hill Book Co., New York, 1917.

PERCIVAL, J. Agricultural Bacteriology. Duckworth & Co., London, 1920.

PRESCOTT, S. C., and WINSLOW, C.-E. A. Elements of Water Bacteriology. John Wiley & Sons, Inc., New York, 1924.

PRUDDEN, T. M. The Story of Bacteria and Their Relations to Health and Disease. G. P. Putnam's Sons, New York, 1910.

RACE, J. The Examination of Milk for Public Health Purposes. John Wiley & Sons, Inc., New York, 1917.

RIDEAL, S., and RIDEAL, E. K. Chemical Disinfection and Sterilization. Edward Arnold, London, 1921.

RODDY, J. Medical Bacteriology. P. Blakiston's Son & Co., Philadelphia, 1917.

ROSENAU, M. J. Preventive Medicine and Hygiene. D. Appleton & Co., New York, 1926.

RUSSELL, H. L., and HASTINGS, E. G. Agricultural Bacteriology. The Century Co., New York, 1921.

SAVAGE, W. G. The Bacteriological Examination of Food and Water. Cambridge University Press, 1914.

SAVAGE, W. G. Food Poisoning and Food Infections. Cambridge University Press, 1920.

SEDGWICK, W. T. Principles of Sanitary Science and the Public Health. The Macmillan Co., New York, 1914.

STEVENS, F. L. Diseases of Economic Plants. The Macmillan Co., New York, 1921.

STITT, E. R. Practical Bacteriology, Blood Work and Animal Parasitology. P. Blakiston's Son & Co., Philadelphia.

TANNER, F. W. Bacteriology and Mycology of Foods. John Wiley & Sons, Inc., New York, 1919.

THOMAS, S. Bacteriolgy: A Text Book on Fundamentals. McGraw-Hill Book Co., New York, 1925.

VAUGHAN, V. C. Protein Split Products in Relation to Immunity and Disease. Lea & Pebiger, Philadelphia, 1913.

ZINSSER, H. Infection and Resistance. The Macmillan Co., New York, 1919.

ZINSSER, H. A Textbook of Bacteriology. D. Appleton and Co., New York.

INDEX